Effects of Rising Temperature on Interactions between Monarch Butterflies and their Parasites

by

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ABSTRACT

During the last few decades, global climate change has been one of the major drivers of species extinction, and has altered many ecological interactions. Monarch butterflies, which represent a cultural icon in North America, have been experiencing population declines due to several anthropogenic forces associated with environmental change. In addition, a monarch protozoan neogregarine parasite, *Ophryocystis elektroscirrha* (OE), aggravates their situation by decreasing the reproductive success and longevity of adult butterflies. Previous studies have shown that global warming can modify the interaction between parasites and their hosts. This study aimed to assess how a rise in temperature affects the virulence and transmission potential of OE. I hypothesized that an increase in temperature would result in shorter adult life spans and higher parasite loads. A total of 154 larvae were reared on artificial diet in incubators at three temperatures, 22°C, 25°C and 28°C until adult emergence or premature death. Approximately 30 larvae from each temperature were inoculated with 10 OE spores and 20 larvae were un-inoculated controls. Developmental time, pupal weight and spore loads were measured and data were analyzed using generalized linear models. Unexpected co-infection with an unknown pathogen resulted in high larval mortality in both OE-inoculated and control treatments, preventing an independent assessment of the effect of temperature on the monarch-OE interaction. As a result of co-infection, monarchs inoculated with OE were more likely to die during the larval stage. As expected, the total developmental time of butterflies declined at higher temperatures. Importantly, only 8 out of 42 OE-inoculated larvae that survived to adulthood produced OE spores, suggesting an antagonistic interaction between the unknown pathogen and OE. Overall, higher temperatures appeared to have a largely

positive effect on monarch performance, since butterflies reared at warmer temperatures had shorter developmental times, resulting in a decrease in exposure time to the unknown pathogen. In conclusion, the unknown pathogen interacted in complex ways with OE, affecting monarch performance more than the increase in temperature itself. In this changing world, temperature sensitive host-parasite interactions could potentially be altered by global warming. However, they need to be studied within the context of how multiple pathogens will interact with their hosts under elevated temperature scenarios.

INTRODUCTION

Over the last century, our planet has been experiencing rapid alterations in climate caused by human activity (Rahmstorf et al. 2007, Smith et al. 2009). Changes in precipitation and temperature across the globe have been shifting the distribution of ecosystems, communities, and populations (Walther et al. 2002, Walther 2010), and the physiology of individuals (Root et al. 2003). For example, alterations in river flow due to modified precipitation regimes can have impacts on entire freshwater ecosystems (Doll and Zhang 2010). Likewise, precipitation variation can shift plant communities and induce alterations in soil ecosystems (Kardol et al. 2010). Moreover, climate change is leading to mismatch in resource and consumer phenology, which causes population declines of animals such as migratory birds (Both et al. 2006). Increases in temperature can also lead to changes in organism traits such as coloration (Root et al. 2003, Roulin 2014). Critically, ecological interactions such as predation, competition, symbiosis and parasitism are also experiencing major alterations under climate change (Hoegh-Guldberg 1999, Tylianakis et al. 2008) and merit increased study.

For example, climate change may alter parasite-host interactions. Rising temperatures can increase parasite virulence and transmission rates and decrease incubation periods. For example, in malaria, the process of sporogony, which consists of the incubation period of the parasite's spores, is temperature-dependent (Detinova 1962). A rise in temperature due to climate change accelerates sporogony, increasing the rate of development of the malaria parasite. Moreover, a rise in temperature can accelerate the development of the mosquito host, increasing the number of bites per life time, which in turn increases transmission rate (Lindsay and Birley 1996).

Another study of malaria in an East African highland associated temperatures from 1970 to 2003 with reported cases of malaria. According to this study, an increment of around 1^{o}C over this period of time was associated with an increase in the size of malaria epidemics, due to an increase in transmission of the disease (Alonso et al. 2011). Furthermore, a recent study in the highlands of Colombia and Ethiopia showed that variation in temperature can affect the spatial-temporal distribution of malaria, by shifting the vector to higher elevations (Siraj et al. 2014). Malaria incidence decreases at high elevation, because lower temperatures reduce the mosquito's reproduction and biting rate and also slow down the development of the parasite. But warmer temperatures due to climate change are leading to a higher altitude range of mosquito vector populations. This could increase the risk of epidemic in highlands where there are dense human populations. Siraj et al (2014) predict an increase of malaria cases in Ethiopia between 35 and 64 percent and between 10 and 80 percent in Colombia when the temperature rises 1°C. In a similar example, research using a gradient in altitude as a proxy for temperature showed that at lower altitude, which coincides with higher temperatures, ticks and their hosts were more abundant. Ticks are vectors for Lyme disease, tick-borne encephalitis virus and loupin ill virus. Ticks' hosts include deer, hare and grouse. If climate change shifts tick and host abundance to higher altitudes, then there will be higher risk of the transmission of these diseases to humans, livestock and wildlife on upland areas. (Gilbert 2010).

Likewise, the interaction between amphibians and the trematode, *Ribeiroia ondatrae,* is temperature-dependent. *Ribeiroia ondatrae,* a planorbella worm, infects three hosts sequentially: snails, amphibians, and mammals. When infecting snails, it

reduces the fecundity by consuming the snail gonads; when infecting amphibians, it creates limb malformations or it atrophies the development of the limbs. When there is a rise in the temperature, the growth rate of both the snail and the parasite are faster, increasing the abundance of the parasite and its transmission to amphibians. Thus, the disease is enhanced by temperature (Paull and Johnson 2011).

On the other hand, a rise in temperature does not always increase the rate of parasite transmission. In the plague, the bacteria, *Yersinia pestis,* enhances transmission by blocking the proventriculus (foregut valve) of the flea vector, stimulating the flea to keep biting in order to get a blood meal. When the temperature rises by more than 5°C, the bacteria is unable to block the proventriculus of the flea, thus the transmission rate (biting rate) declines (Hinnebusch et al. 1998).

Some parasites that have a portion of their life cycle outside a host, and depend on water for transmission, may be spread more easily under global climate change (Slenning 2010). A study of how climate change can influence water-related diarrheal diseases, such as cholera, showed that an increment in temperature of approximately 1ºC in Tanzania from 1977 to 2004, was associated with a higher number of cholera cases because of increasing rainfall. The dry season was negatively associated with the number of cholera cases, since cholera depends on water for transmission. The predictions from this research for 2030 describe a scenario with a 15 to 30 percent increase in cholera cases in a model using an increment of 1°C, and between 60 and 100 percent, when considering an increment of 2°C (Traerup et al. 2011).

Based on all the cases mentioned above, it appears critical to keep conducting research that evaluates how disease dynamics are altered under changing climate

regimes. Therefore, the overall goal of my research was to increase our understanding of disease dynamics between a parasite and its host in a changing environment. Such work can help to predict the impact of disease on humans, livestock and wildlife as the temperature continues to increase on our planet. I studied the effects of climate change on disease dynamics using the protozoan parasite, *Ophryocystis elektroscirrha* (OE for short), and the monarch butterfly, *Danaus plexippus*.

Study system

The monarch butterfly, *Danaus plexippus*, (Lepidoptera: Danaidae) and its OE parasite are a good study system for several reasons. First, *O. elektroscirrha* belongs to an apicomplexan group called Gregarinia, which is related to the protist *Plasmodium*, which causes malaria, and to *Toxoplasma,* which causes toxoplasmosis. Both of these diseases can prove lethal to humans. Therefore, by studying how the transmission rate and virulence of this parasite change in different temperature scenarios, general predictions for other diseases caused by similar types of parasites might be made. Moreover, there is a good foundation of information collected for more than 70 years on monarch butterflies (Beall 1941, Brower 1961, Reichste et al. 1968). This amount of information makes research on this organism easier. Furthermore, because monarch butterflies are a threatened species due to habitat loss and OE parasite infection (McLaughlin and Myers 1970, Pleasants and Oberhauser 2013), research related to their diseases is critical, in order to help understand potential conservation efforts. Likewise, since monarch butterflies are iconic in North American culture, can relate to their conservation and protection (Pleasants and Oberhauser 2013, Diffendorfer et al. 2014,

Flockhart et al. 2015). Additionally, both the parasite and host can be easily manipulated for experimentation. For example, the number of parasites that infect the monarch caterpillars can be controlled, and it is easy to quantify adult survival and fitness.

Monarchs have a metamorphic life cycle comprised of four stages: egg, larva (caterpillar), pupa (chrysalis) and adult butterfly. The larva first consumes its own eggshell, and then feeds on milkweed plants (*Asclepias sp*.) until reaching pupation. When the adult emerges, its main source of energy comes from nectar. In eastern North America, monarchs migrate to Mexico to overwinter and then return for the breeding season in summer.

Monarch butterflies worldwide are infected by a neogregarine parasite, *Ophryocystis elektroscirrha* (McLaughlin and Myers 1970, Altizer et al. 2000) (Figure 1). *O. elektroscirrha*, OE for short, infects the caterpillar when an infected butterfly lays eggs covered with the parasite spores and also spreads spores on milkweed leaves during oviposition. The neonate larva gets infected when it eats the eggshell or surrounding foliage, introducing this parasite into its mid gut, where OE spores lyse and release sporozoites that cross the midgut wall reaching the hypodermis. The parasite then reproduces asexually and when the caterpillar goes into the pupal stage, the parasite reproduces sexually. Finally, virulence is expressed after the spore-covered adult emerges from the chrysalis, reducing monarch flight ability, fecundity and lifespan (Altizer and Oberhauser 1999, de Roode et al. 2007). Population models suggest that OE may reduce the size of some monarch populations by 50% (Altizer et al. 2004).

As previously stated, studies linking global climate change and disease dynamics should be conducted to better predict their interactions in a changing world. Therefore my research proposed to assess how temperature affects the disease dynamic of monarch butterflies and their parasite. I hypothesized that a rise in temperature will increase the virulence and transmission potential of the OE parasite. Based on climate change models that expect 1°C to 3°C increases in temperature (IPCC 2013), I predicted that larvae and pupae reared at higher temperatures will suffer an increase in both virulence and transmission potential compared with butterflies reared at ambient temperatures. A decrease in pupal mass and adult lifespan denotes an increase in virulence (de Roode et al. 2008b), while a higher parasite load denotes as increase in transmission potential (de Roode et al. 2008b, de Roode et al. 2009).

MATERIALS AND METHODS

Temperature experiment

To test the effect of temperature on virulence and transmission potential of the OE parasite on *D. plexippus,* I reared monarch larvae to the second instar at one of three different temperatures and then infected them with OE spores. Larvae were then returned to their experimental temperature and reared until adult eclosion or death. Some models predict temperature increases of up to 3°C (IPCC report 2013, Leung and Qian 2005, Di Lorenzo 2015) and I based my experiments on this value. Given the wide latitudinal distribution of monarch breeding in the US, I chose to compare three temperatures that correspond to summer breeding temperatures at high, medium and low latitudes. The temperatures chosen were selected using July daily average

temperature of three cities that are located on each of those three latitudes and that approximate the distribution of monarchs in the eastern US. Each of those temperatures differs by 3°C. That way the mid latitude temperature serves as the potential climate change scenario of high latitude monarch breeding sites. Likewise, the low latitude temperature serves as the potential climate change scenario of mid latitude monarch breeding sites. Specifically, the high latitude temperature is 22.6°C; the mid latitude temperature is 25.8°C, and the low latitude temperature is 28.9°C. For example, a comparison based on the temperatures of high and mid latitudes can assess (a) current differences in parasite virulence and transmission potential between these breeding points, and (b) future expectations for high latitude breeding sites.

I infected a total of 30 monarch caterpillars per temperature treatment, and I had an additional 20 caterpillars as uninfected controls for each temperature. Inoculations occurred over a period of 12 days as neonates became available; larvae were divided evenly among treatments each day. Monarch eggs for this experiment were obtained from a colony raised in our lab. This colony originated from a migratory monarch population in the Midwestern US. For my experiments, I obtained eggs from four to six female butterflies kept inside net enclosures, fed *at libitum* with a honey solution on sponges. Eggs were laid on *Asclepias tuberosa* and *Asclepias curassavica* plants. Neonate caterpillars were assigned randomly to treatment groups and placed in individual 1 oz. plastic cups immediately after hatching. I raised all the caterpillars in individual cups, inside incubators at their corresponding temperature, all at 16:8 light:dark. Because larval host plant quality can influence the virulence and transmission potential of OE (de Roode et al. 2008a, Sternberg et al. 2012), I reared all

monarchs on artificial diet throughout their development to equalize larval diet quality among temperatures. However, to ensure that each inoculated larva received the same dose of OE spores, I infected treatment larvae by placing 10 parasite spores on one *Asclepias tuberosa* (butterflyweed) leaf disc (8mm diameter) that was consumed by each treatment larva. All disks came from a single *A. tuberosa* plant. The foliage of *A. tuberosa* is free of the cardenolides that influence monarch infection by OE. To ensure consumption of all parasites, I enclosed each 2nd instar larva in a petri-dish with a leaf disk inside; only caterpillars that consumed their entire leaf disk (and therefore entire parasite dose) were used in the experiment. The OE spores for my experiment originated from a single butterfly from a Georgia population.

Measures of virulence & transmission potential

For holometabolous insects, pupal mass is often tightly correlated with fecundity (Lill and Marquis 2001). 72 hours after pupation, I weighed pupal mass as a proxy for fecundity. Therefore, a decrease in pupal mass associated with OE infection served as a measure of virulence (decline in fitness). I determined transmission potential by the number of spores carried by the butterflies (parasite load). I estimated transmission potential (parasite load) by counting spores on adults (de Roode et al. 2009). Spore loads were quantified by vortexing monarch bodies in 5 ml of water and counting the number of spores using a hemocytometer slide. Finally, I measured adult lifespan as a second index of virulence. After adult eclosion, I placed butterflies in glassine envelopes at 14^{o}C and monitored them daily to calculate the number of days between eclosion and death (de Roode et al. 2007).

Monarch realized production estimate

In the middle of the experiment, the caterpillars became exposed to a second unknown pathogen that interacted with OE and temperature in complex ways (see "Results", below). In order to understand how co-infection and temperature influenced both survival and fitness we made a simple calculation. We multiplied the percent monarch survival under each treatment by the average mass of the survivors to obtain monarch realized production (realized biomass). This was calculated for females and for males independently, and then they were summed to calculate "total production." We could then calculate the effect of temperature on OE virulence (in the presence of co-infection) by subtracting average inoculated monarch production from the average monarch production of controls.

Plant chemistry and wing chemistry

I estimated the foliar cardenolide concentration of the plant used as the source of leaf disks (above) to ensure that it was free of cardenolides. I collected three leaf samples from the foliage of the plant and analyzed them for cardenolide concentration. Briefly, foliage for cardenolides was extracted in methanol and analyzed using high performance liquid chromatography with digitoxin as an internal standard, using well-established methods (de Roode et al. 2008a, Sternberg et al. 2012, Tao et al. 2015). I also analyzed ten cups of artificial diet to confirm the absence of cardenolides in the diet, which is based on agar, nutrients, vitamins and low-cardenolide milkweed powder. I also analyzed the artificial diet for carbon and nitrogen concentration using a TruMac

CN Analyzer. (Leco Corporation, St. Joseph, MI). Finally, I analyzed the wings of all surviving butterflies to determine if there was any trace of cardenolide sequestered from the artificial diet.

Statistical Analysis

All analyses were conducted using SAS 9.4. The proportion of larvae that died before maturity was compared among temperature treatments using generalized linear models with a binomial distribution and a logit link function (Littell et al. 2002). A similar model was used to compare the proportion of inoculated larvae that produced spores as adults among temperature treatments. General linear models were used to compare the following indices of monarch performance among temperature treatments; larval period, pupal period, adult period, total developmental period, and pupal mass. Because all experimental larvae were not inoculated on the same day, we used the Julian date of inoculation as a covariate to account for any variation in egg or diet quality over time. The first inoculation started on November first (Julian day 305) and the last inoculation was on November 20th (Julian day 324).

RESULTS

We observed an uncontrolled infection with an unidentified pathogen that altered the interaction between OE and monarch butterflies. This co-infection occurred in both control and inoculated monarchs, and may have prevented a clear assessment of how temperature affects OE-monarch parasitic interactions. Effects of the unknown

pathogen were most apparent as caterpillars began the process of pupation (Figure 3). Symptoms included an extended larval period, lethargic behavior, mortality entering or during the pupal stage, and suppuration of a black fluid after death. Contamination with the unknown pathogen increased as the experiment progressed, so that later inoculations were more likely to suffer co-infection than were early inoculations. While I recognize that this co-infection has compromised the original experimental design, the results presented below nonetheless provide insight into how OE and co-infection influence simultaneously monarch butterflies under a range of experimental temperatures.

Mortality

Monarch mortality varied between 26% and 63% among treatments (Figure 4). Larvae inoculated with OE were marginally more likely to die before adult emergence than were uninoculated larvae (X^2=3.26, d.f.=1, *P*=0.0709, Figure 4). The high mortality in the control larvae represents the presence of the additional pathogen, which appears lethal to monarchs. Monarch mortality was marginally higher at 25°C than at 22 or 28°C (X^2 = 4.86, d.f.= 2, *P*=0.0882, Figure 4), but temperature had no effect on the magnitude of mortality increase caused by OE inoculation (inoculation by temperature interaction: X^2 = 1.00, d.f.= 2, *P* =0.6068, Figure 4).

Effects of increased temperature on OE virulence

(1) Monarch pupal mass

Overall, inoculated pupae weighed more than uninoculated pupae, and pupal mass increased with temperature ($F_{1,61}$ = 6.72, P=0.0119, $F_{2,61}$ = 3.87, P=0.0262, respectively, Figure 5). Notably, female pupal mass (and therefore fecundity) was particularly responsive to increasing temperature (Inoculation*temperature*gender $F_{2,61}$=4.88, P=0.0108, Figure 5). However, female pupae reared at 28 ^{o}C weighed less when inoculated than when uninoculated, although sample size was low for this group of pupae. Despite these overall trends, individuals inoculated later in the experiment actually weighed less than their counterpart control pupae, suggesting an interaction between OE and increasing contamination by the unknown pathogen ($F_{1,61}$ = 6.68, P = 0.0122, Figure 6a). Moreover, monarch pupal mass declined markedly at lower temperatures during later inoculations, presumably because slower development increased exposure to contamination ($F_{2,61}$ = 3.90, P = 0.0255, Figure 6b)

(2) Monarch lifespan and developmental period

Neither temperature nor infection by OE influenced adult lifespan (inoculation $F_{1,60}$=0.98, P=0.3267, Temperature $F_{2,60}$=2.62, P=0.0807, inoculation*temperature interaction $F_{2,60}$=0.96, P=0.3864, Table 1). However, larval period, pupal period and total developmental period of the butterflies were reduced at higher temperatures as expected for a poikilotherm ($F_{2,70}$=11.45, P <0.0001, Table 1).

Overall, inoculated caterpillars grew faster (shorter larval period) than did uninoculated ones ($F_{1,70}$=7.88, P=0.0065, Figure 7a). This pattern became more pronounced for caterpillars that were inoculated later (inoculation*inoculation date: $F_{1,70}$= 7.97, P=0.0062). As expected, larvae reared at warmer temperatures grew faster ($F_{2,70}$=11.45 ,P < 0.0001, Figure 7b), with temperature-mediated differences in larval

period increasing as the experiment progressed (temperature*inoculation date $F_{2,70}$= 11.84, P <0.0001). This likely arises as increasing contamination magnified the differences in larval period caused by temperature (Figure 7b). The length of the pupal period decreased with increasing temperature ($F_{2,72}$=97.94 ,P < 0.0001, Figure 8) but was unaffected by inoculation with OE.

Over the entire developmental period, butterflies infected with OE had shorter life cycles than did control butterflies ($F_{1,67}$ =5.02, P=0.0284, Figure 9). Furthermore, as expected, butterflies growing at warmer temperatures had shorter developmental periods ($F_{2,67}$=7.04, P =0.0017, Figure 10). As noted previously, individuals that were inoculated later with OE expressed even longer life cycles (temperature*inoculation date $F_{2,67}$=7.20, P=0.0015), presumably as a result of increasing co-infection.

Effects of increased temperature on OE transmission potential

Of the 42 inoculated butterflies that survived to adulthood, only 8 produced OE spores and I was therefore unable to compare spore loads among treatment groups. However, the likelihood of producing any spores differed marginally among temperatures, whereby the probability of producing E spores appeared much higher at 22^{o}C than at higher temperatures (X^2=5.27, d.f.=2, P=0.0716, Figure 11). We suggest that co-infection with the unknown pathogen greatly reduced spore production at higher temperatures.

Chemical analysis of artificial diet and butterflies

The artificial diet that I used averaged 42.74% carbon and 6.35% nitrogen. The C:N ratio was 6.73, similar to values measured in the artificial diets we have used in previous laboratory experiments (carbon: 40%, nitrogen: 6%, C:N = 6.57). There were no measurable cardenolides present in the artificial diet. Likewise, the *A. tuberosa* plants that served as sources for the leaf disks we used during inoculation contained no measurable cardenolides. Finally, none of the butterfly wings that we analyzed contained measurable cardenolide concentrations.

Monarch realized production estimate

OE-inoculated females had lower realized production than did control females (Figure 12A) . The difference in production between inoculation treatments was particularly pronounced at the highest temperature of 28°C, at which the production gained was around three times higher than the production gained at 22°C and 25°C. The same pattern was observed for males (Figure 12B) and for total monarch production (Figure 12C).

OE-virulence increased markedly at 28°C (Figure 12D) because infection with OE eliminated all biomass gains of control monarchs at that temperature.

DISCUSSION

In this study, I found that co-infection with OE and another (unknown) pathogen altered monarch butterfly performance and longevity. Importantly, I have shown that

the effect of this co-infection on monarch performance may be greater than the effect of the predicted rise in temperature associated with global warming. However, I observed some complex interactions between co-infection and temperature treatment.

First, and perhaps the most important finding, this additional pathogenic burden can result in high mortality at larval, pupal and adult stages of monarch butterflies. The highest mortality rates were observed in larvae, particularly when they were infected with OE (Figure 4). This is especially interesting because OE is usually not lethal to monarchs, reducing only the lifespan and fitness of adult hosts (McLaughlin and Myers 1970, de Roode et al. 2008a, de Roode et al. 2008b). At this time, I have not been able to identify the unknown pathogen. In addition to OE, monarch butterflies have been reported to suffer infection from nuclearpolyhedrosis virus, *Nosema* (a microsporidian), the bacterium *Micrococcus flaccidifex danai*, and *Pseudomonas* bacteria (Brown 1927, McLaughlin and Myers 1970)). Although I sent monarch cadavers to the insect pathology imaging group at Mississippi State University, they were unable to isolate any of the known pathogens of monarch from my samples.

Humans and other animals often experience co-infection by different pathogens like bacteria, viruses, nematodes, fungi and macro-parasites among others, and may even suffer from three or more infectious diseases simultaneously (Lau et al. 2010, Romansic et al. 2011). These co-infections might act in a synergistic way, increasing virulence (Romansic et al. 2011) or in an antagonistic way, obstructing the development or resource intake of the other pathogen (Fenton et al. 2008, Jolles et al. 2015). Finally, agents of co-infection can coexist without having effects on each other (Pedersen and Fenton 2007).

When discussing synergism or antagonism, it is important to distinguish between effects on the fitness of the host and effects on the fitness of the parasite. For example, the secondary pathogen present in my caterpillars interacted in an antagonistic way with OE. As described above, those individuals infected with OE were more likely to die before adulthood than were individuals not infected with OE (Figure 4). This co-infection might be synergistic for the unknown pathogen, but is clearly antagonistic for OE, which requires mobile adult butterflies for transmission (Figure 1).

I observed a second powerful indication of the antagonism between the unknown pathogen and OE. Of the monarchs that I inoculated with OE that survived the co-infection, only 19% (8 out of 42 survivors) produced any OE spores. Typically, nearly all inoculated larvae develop into adults with substantial spore loads (Sternberg et al. 2012). This important result demonstrates that adult monarchs that survived the co-infection should not represent a high risk of OE transmission to other individuals. However, the unknown pathogen can cause substantial monarch mortality (Figure 4), overwhelming any potential positive effects of clearing the OE parasite.

What might have led to such high pathogen burdens in my experimental monarchs? My chemical analysis of the artificial diet showed that the diet was free of cardenolides and perhaps other important secondary metabolites that might control pathogens in wild monarch populations (de Roode et al. 2013). Previous work on the gypsy moth, *Lymantria dispar*, has shown that the LD50 of the gypsy moth nuclear polyhedrosis virus is 800 polyhedral inclusion bodies (PIBs) on artificial diet whereas it is closer to 50,000 PIBs on foliage (Hunter 2016). It is therefore possible that the use of artificial diet magnified the interaction between the unknown pathogen and OE. However, artificial diet was not the cause of co-infection, as artificial diet has been used

in numerous other experiments and can successfully maintain healthy colonies during their larval cycle. Moreover, similar symptoms of an unknown disease agent have been reported from foliage-feeding monarchs in other research labs and in natural monarch populations. Nonetheless, all previous studies of OE transmission potential and virulence have used fresh plant foliage (de Roode et al. 2008a, Sternberg et al. 2012, Tao et al. 2015). Milkweed foliage has important anti-pathogenic properties (de Roode et al. 2008a) and mediates variation in the gut microbiome of monarch caterpillars (M.D. Hunter & J. de Roode, unpublished work). Additionally, the rearing conditions that I used could have enhanced the exposure or virulence of the unknown pathogen. Specifically, while larvae were reared in closed individual cups, lower levels of ventilation and higher levels of humidity may have increased the potential for infection. Moreover, despite sterilizing all equipment between each individual feeding, there exists the potential for contamination among cups within incubators.

Probability of co-infection with the unknown pathogen may have increased over the course of the experiment. This may explain the increasingly deleterious effect of OE inoculation on monarch pupal mass in later inoculations (Figure 6a). Moreover, the development times of larvae and pupae became longer in later inoculations, again suggesting an increase in infection by the unknown pathogen (Figures 7, 9, 10).

Given that my study was originally designed to investigate the effect of temperature on monarch-OE interactions, I am particularly interested in how my temperature treatments influenced monarch performance under co-infection. It appears that the unknown pathogen modified the effects of temperature on monarch growth rate and pupal mass. As expected, larvae and pupae developed faster at warmer than at cooler temperatures (Figures 7b, 8). However, at 22^{o}C, larval development

became notably slower in later inoculations, presumably as a result of more contamination (Figure 7b). This same pattern was evident in the total developmental period of monarchs, whereby individuals that were inoculated later in the experiment grew particularly slowly at cooler temperatures (Figure 10). Additionally, pupal mass decreased in later inoculations, especially at cooler temperatures (Figure 6b). I infer from this that warmer temperatures, and the shorter larval periods that they caused, helped to protect monarch larvae from co-infection and its deleterious consequences for pupal mass. However, I also observed that surviving monarchs were more likely to produce OE spores at lower than at higher temperatures (Figure 11), suggesting that high temperatures increased the antagonistic effects of the unknown pathogen against OE if co-infection had occurred.

My observations of monarch responses to temperature are broadly consistent with previous studies. For example, (Zalucki 1982) reported that the optimal temperature for larval survival and growth in monarchs is from 27 to 29° C. Temperatures higher than 30° C or lower than 25° C slow larval development considerably, and temperatures above 33 and under 12° C are lethal to caterpillars. Moreover, variation in temperature with alternating periods of high temperature can increase developmental rate and decrease the probability of exposure the pathogens (Zalucki 1982, York and Oberhauser 2002). York and Oberhauser (2002) also reported that monarchs weighed less after exposure to high temperatures, which is in contrast to my results; in my study, monarch pupal masses were generally greatest under my highest temperature treatment (Figure 5). However, York and Oberhauser (2002) used an extreme high temperature treatment of 36 ^{o}C in their experiments, greatly exceeding the optimal temperature for monarchs, and higher than my highest treatment of 28 ^{o}C.

Overall, based on my work and previous studies (Zalucki 1982, York and Oberhauser 2002, Lemoine 2015, Lemoine et al. 2015), higher temperatures within the optimal range for monarchs appear to increase butterfly performance and likely decrease the probability of pathogen infection.

My study focused on isolating the effects of temperature on monarch-parasite interactions, keeping food quality constant on an artificial diet. In the field, it is possible that changing plant chemistry under rising temperatures might influence plant quality, altering monarch-OE interactions. Previous studies investigating how temperature influences milkweed foliar chemistry provide contradictory results. For example, Lemoine et al. (2015) reported that warming had no influence on specific leaf area, water content, or latex exudation of milkweed leaves, concluding that rising temperatures would have no effect on foliar quality for monarchs. In contrast, Couture et al. (2015) reported increases in foliar nitrogen concentration and decreases in foliar thickness with increasing temperature; both were associated with improved monarch performance. However, Lemoine et al. (2015) used a lower range of temperatures (12 to 15 ^{o}C) than did Couture et al. (2015) (24 to 30 ^{o}C). The latter study was therefore similar to ours in its temperature range. While these plant traits may influence monarch susceptibility to the unknown pathogen, they are unlikely to influence OE infection, which is associated primarily with foliar cardenolide concentrations (de Roode et al. 2008a, Gowler et al. 2015). Rather, rising temperatures will likely have a greater influence on the future distribution of milkweed species (Lemoine 2015). Milkweed species vary enormously in their cardenolides (Agrawal and Fishbein 2008) and their antibiotic effects on monarch parasites (Sternberg et al. 2012).

I found one previous study of the effects of temperature on the virulence and transmission of OE in monarchs. This unpublished doctoral dissertation (E.A. Lindsey, Emory University, 2008) suggests that infected monarchs reared at lower temperatures produced fewer spores while monarchs reared at higher temperatures suffered higher virulence. This unpublished work generally contradicts my findings, in which spore loads were greatest at lower temperatures (Figure 11) and monarch pupal mass increased with temperature (Figure 5). The different results from these studies may stem from the prevalence of co-infection in my experiments, or the use of milkweed foliage rather than artificial diet in the experiments by Lindsey. Clearly, additional work will be necessary to isolate the precise effects of temperature on monarch-OE interactions.

Another unexpected result in my experiment was that inoculated and control adult butterflies had similar lifespans. This is surprising because OE is known to significantly decrease adult longevity (McLaughlin and Myers 1970, de Roode et al. 2007). However, OE-induced reductions in lifespan are usually associated with high spore loads on adult butterflies. In my experiment, very few of the inoculated butterflies that survived to adulthood produced any spores (see above). It therefore is possible that clearing of OE by the unknown pathogen uncoupled the typical relationship between OE inoculation and adult longevity. Additionally, the most heavily-infected monarchs may have died prior to adult eclosion (Figure 4).

To my knowledge, this is the first study to describe the effects of elevated temperature on co-infection of monarch butterflies with interacting pathogens. Overall,

elevated temperature in combination with a second pathogen affected OE-monarch interactions.

The temperatures chosen for this experiment correspond to three US cities. Specifically, Ann Arbor, MI was our model high latitude city, at ~42 ^{o}N latitude, with a July average daily temperature of 22.6^{o}C; the mid latitude city was Knoxville, TN, at ~35 ^{o}N latitude, with a July average daily temperature of 25.8^{o}C; the low latitude city was Miami, FL, at ~25 ^{o}N latitude, with a July average daily temperature of 28.9^{o}C. Accordingly, Knoxville serves as a model for a "future Ann Arbor" whereas Miami serves as a model for a "future Knoxville." However, the cities also provide predictions for current latitudinal variation in response to co-infection. Hence, we predict that monarch co-infection in Miami will result in higher OE virulence than will monarch co-infection in Knoxville or Ann Arbor. To our knowledge, co-infection has not been studied previously at these locations.

Notably, the higher OE virulence that we observed at higher temperature resulted from co-infection eradicating the gains in performance made by control monarchs at 28^{o}C. The high performance of monarchs at 28^{o}C is consistent with previous research, in which monarch fitness is maximized between 27-29 ^{o}C (Zalucki 1982, Lemoine et al. 2015). On the other hand, the cost of hosting OE (OE virulence) was also higher at 28^{o}C, presumably because all the production gained for controls at 28^{o}C was completely eliminated by OE. Thus, there was a higher loss in monarch production (higher virulence) at the highest temperature. This demonstrates that virulence is not only intrinsic to the OE parasite, but is also influenced by the environmental conditions under which the parasite and host develop.

These results have important implications in a changing world, in which global warming can alter host-parasite interactions. There are, of course, additional potential effects of environmental change on monarch-parasite interactions. For example, the propensity of monarchs to migrate may decline at higher temperatures because of better conditions for larvae and year-round availability of milkweeds (Oberhauser and Peterson 2003). Low migration selects for higher parasite virulence in the OE-monarch system (Altizer et al. 2000). Additionally, environmental change may influence the geographic distribution of parasites, monarchs, and milkweeds (Zipkin et al. 2012, Lemoine 2015) and therefore the complex interactions among them (Hunter 2016). Given the potential expansion or shift in the range of monarchs, they may come into contact with new pathogens, and thus experience novel co-infections. Further work on co-infection under environmental change is urgently needed to predict and manage the populations of organisms in a changing world.

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FIGURE LEGENDS

Figure 1. Lifecycle of the monarch butterfly, *Danaus plexippus*, and its protozoan parasite, *Ophryocystis elektroscirrha*.

Figure 2. Design of an experiment to assess the effects of temperature on the interaction between monarch butterflies and their protozoan parasite. Numbers refer to the sample sizes of caterpillars reared in each treatment group.

Figure 3. Consequences of infection with an unknown pathogen that infected experimental monarch larvae. Caterpillars became lethargic and often died immediately before or during pupation. A black substance suppurating out of their body was usually found. Pupal cases showed deformities and black or brown spots different from those seen when infected just by OE parasites. Most such pupae failed to emerge.

Figure 4. Mortality of monarch butterflies reared at three different temperatures that were either inoculated with OE protozoan parasite or left un-inoculated (control). Initial sample sizes are reported in Figure 2. Temperatures reported are in centigrade.

Figure 5. Pupal mass of female and male monarch butterflies reared at different temperatures, with or without parasite inoculation. “Inoc” are those pupae from larvae that were inoculated with the OE parasite; “Cont” are pupae from un-inoculated larvae. Sample size is reported in Table 1. Bars represent the standard errors around the mean.

Figure 6. The pupal mass of monarchs (A) that were either inoculated or not inoculated with OE parasites, and (B) that were reared under three different experimental temperatures. Julian days refer to the date upon which larvae were originally inoculated with OE.

Figure 7. The larval period of monarchs (A) that were either inoculated or not inoculated with OE parasites, and (B) that were reared under three different experimental temperatures. Julian days refer to the date upon which larvae were originally inoculated with OE.

Figure 8. The pupal period of monarchs that were either inoculated or not inoculated with OE parasites, and that were reared under three different experimental temperatures. Sample size is reported in Table 1. Bars represent standard errors around the mean.

Figure 9. The total developmental period of monarch butterflies that were either inoculated or not inoculated with OE parasites. Julian days refer to the date upon which larvae were originally inoculated with OE. Total developmental period ranges from the day larvae hatched to the day that adult butterflies died.

Figure 10. The total developmental period of monarch butterflies that were reared under three different experimental temperatures. Julian days refer to the date upon which larvae were originally inoculated with OE. Total developmental period ranges from the day larvae hatched to the day that adult butterflies died.

Figure 11. The proportion of OE-inoculated monarch adults that produced OE spores. Monarchs were reared under three different experimental temperatures. Interaction with an unknown pathogen may have cleared OE infection or heavily co-infected individuals may have died before adulthood.

Figure 12. The realized production (A, B, C) of control and inoculated monarch butterflies reared under three different temperatures, and (D) the corresponding virulence (control production minus inoculated production) of the OE parasite at those temperatures. Realized production is calculated as the product of monarch survival and the pupal mass of survivors.

FIGURES

Figure 1

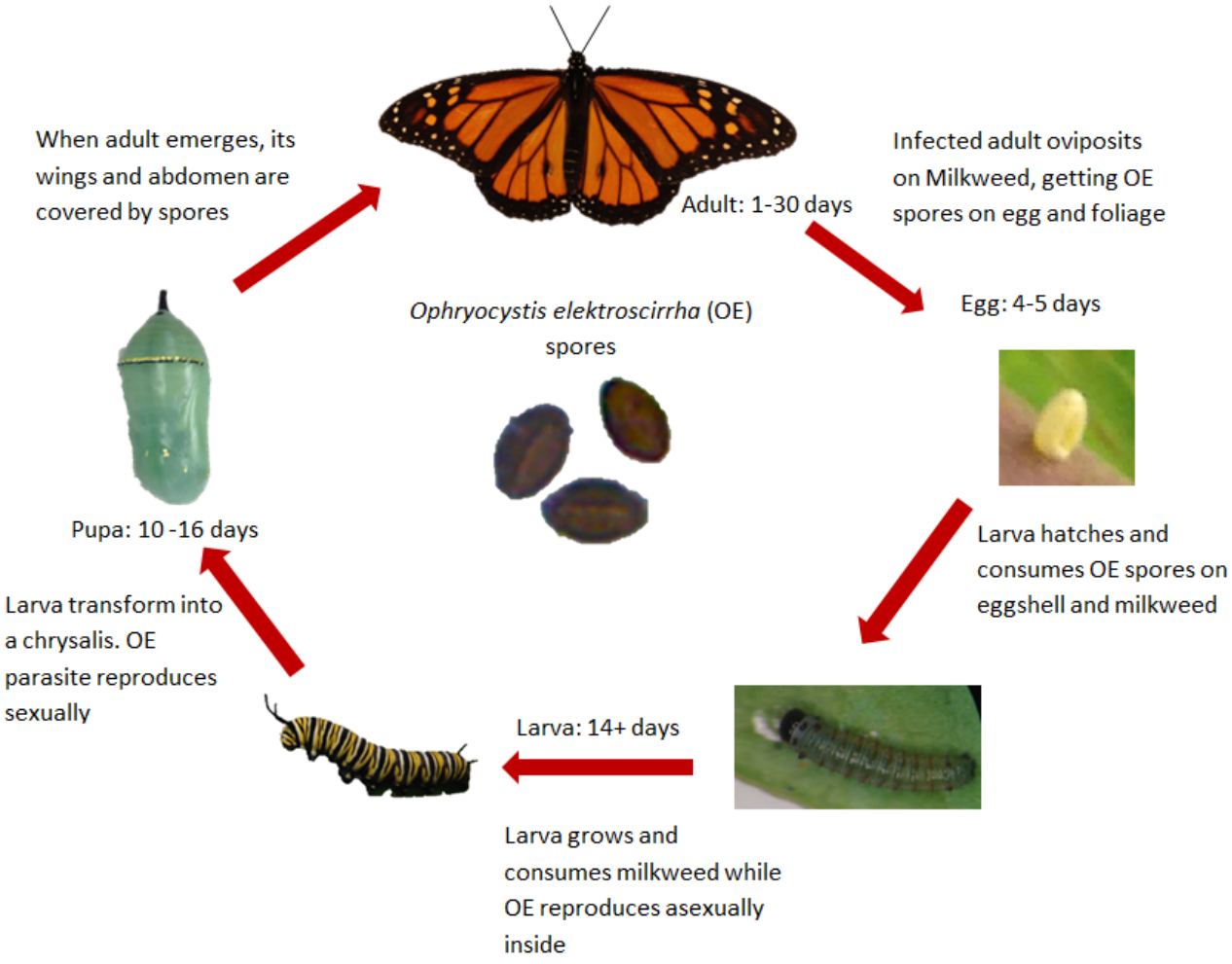


Figure 2

	Ann Arbor, MI		Knoxville, TN		Miami, FL	
	22.6°C		**25.8°C**		**28.9°C**	
	Inoculated	**Control**	**Inoculated**	**Control**	**Inoculated**	**Control**
Number of samples	30	20	32	22	31	19
Total	50		54		50	

Figure 3

Figure 4

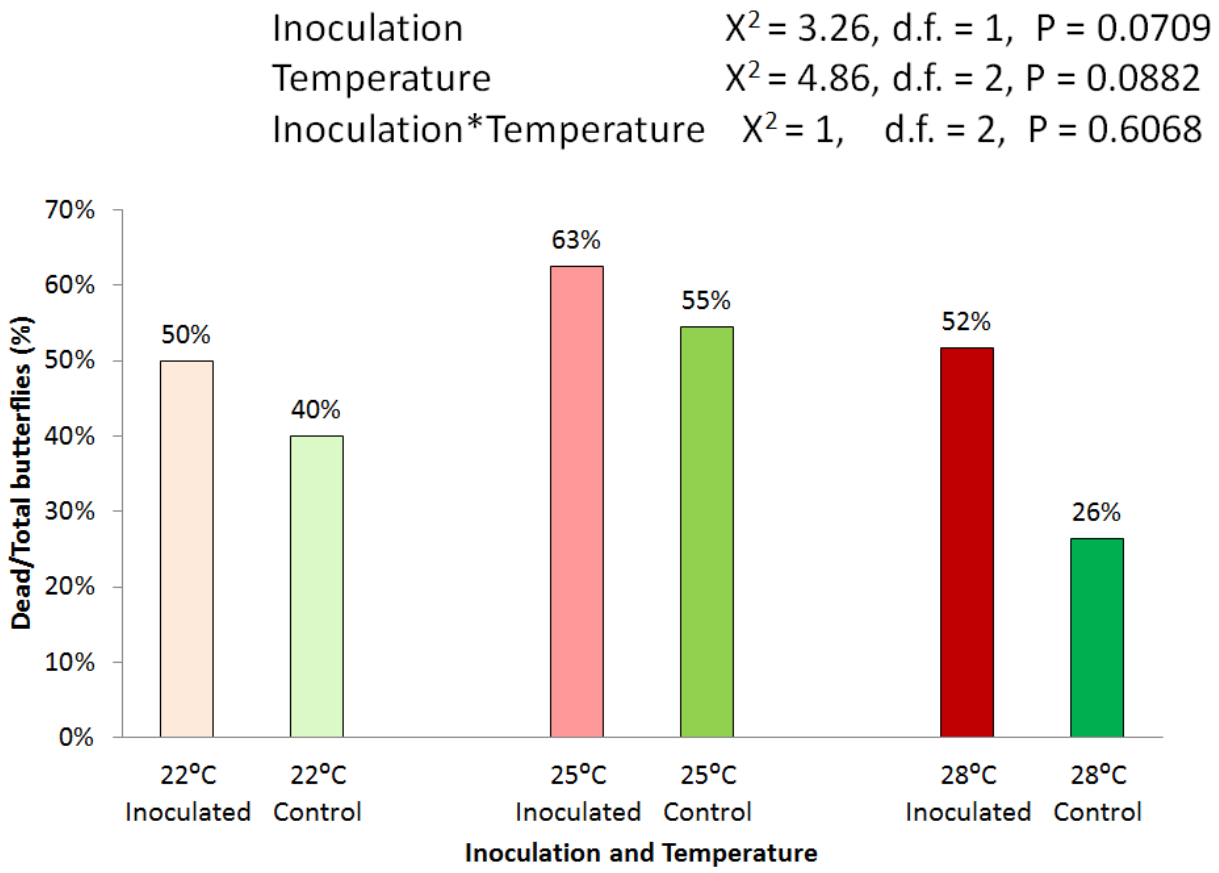

Inoculation $X^2 = 3.26$, d.f. = 1, P = 0.0709
Temperature $X^2 = 4.86$, d.f. = 2, P = 0.0882
Inoculation*Temperature $X^2 = 1$, d.f. = 2, P = 0.6068
70%
60%
50%
40%
30%
20%
10%
0%
Dead/Total butterflies (%)
50%
40%
63%
55%
52%
26%
22°C Inoculated
22°C Control
25°C Inoculated
25°C Control
28°C Inoculated
28°C Control
Inoculation and Temperature

Figure 5

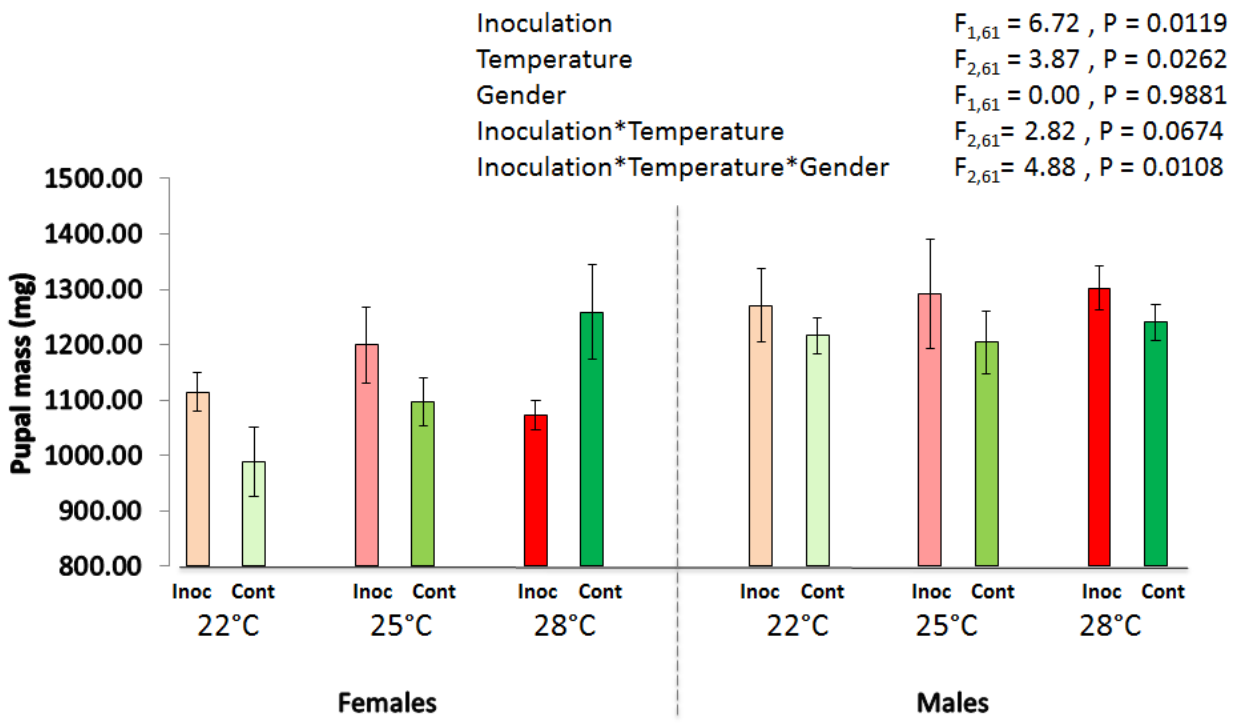
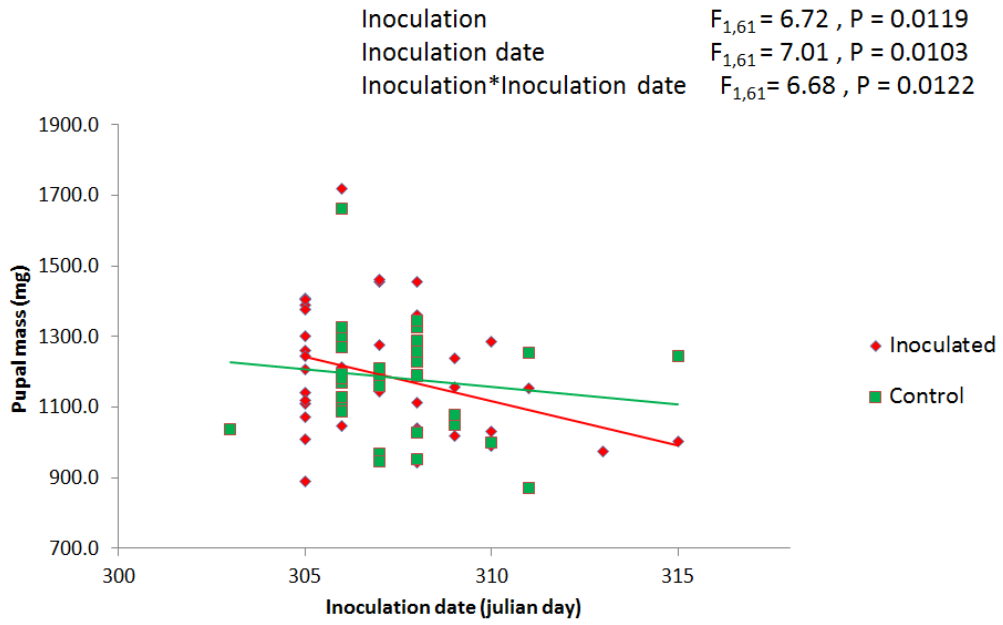

Inoculation
Temperature
Gender
Inoculation*Temperature
Inoculation*Temperature*Gender
$F_{1,61}$ = 6.72 , P = 0.0119
$F_{2,61}$ = 3.87 , P = 0.0262
$F_{1,61}$ = 0.00 , P = 0.9881
$F_{2,61}$= 2.82 , P = 0.0674
$F_{2,61}$= 4.88 , P = 0.0108
Pupal mass (mg)
1500.00
1400.00
1300.00
1200.00
1100.00
1000.00
900.00
800.00
Inoc Cont
22°C
25°C
28°C
Females
Males

Figure 6

A.


Inoculation $F_{1,61}$ = 6.72 , P = 0.0119
Inoculation date $F_{1,61}$ = 7.01 , P = 0.0103
Inoculation*Inoculation date $F_{1,61}$ = 6.68 , P = 0.0122
1900.0
1700.0
1500.0
1300.0
1100.0
900.0
700.0
Pupal mass (mg)
300
305
310
315
Inoculation date (julian day)
Inoculated
Control

B.

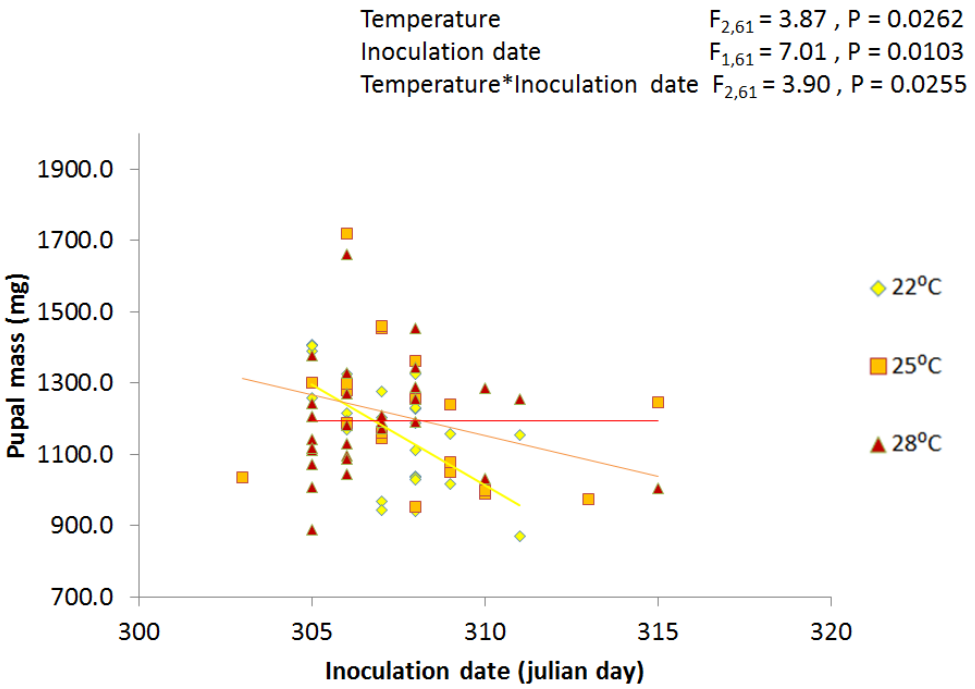
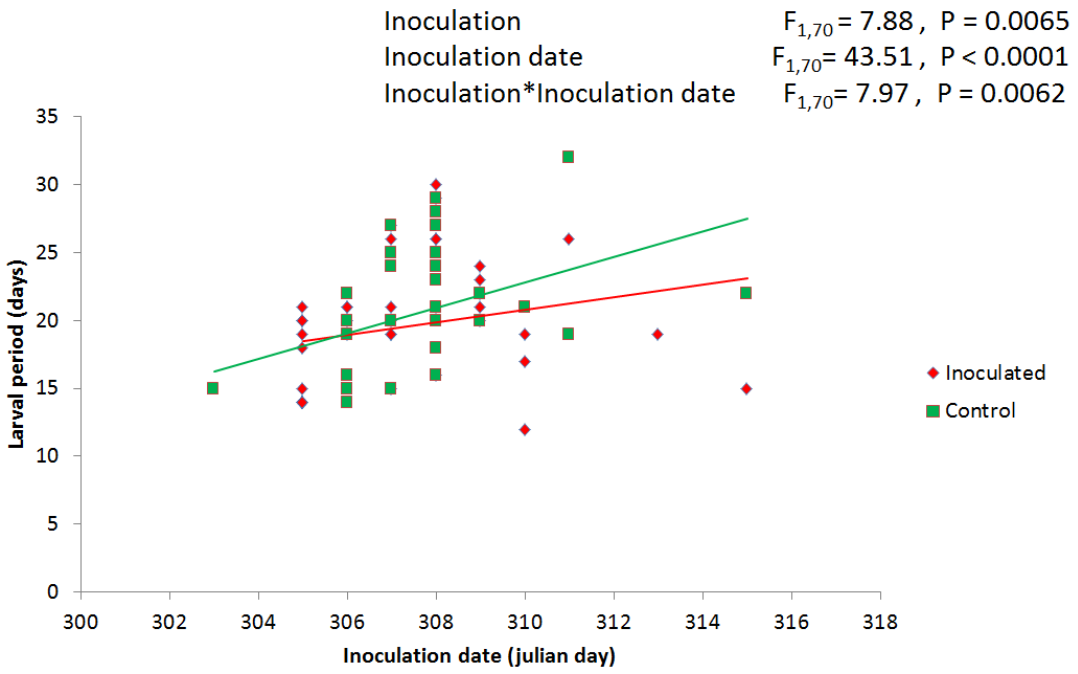

Temperature $F_{2,61}$ = 3.87 , P = 0.0262
Inoculation date $F_{1,61}$ = 7.01 , P = 0.0103
Temperature*Inoculation date $F_{2,61}$ = 3.90 , P = 0.0255
1900.0
1700.0
1500.0
1300.0
1100.0
900.0
700.0
Pupal mass (mg)
300
305
310
315
320
Inoculation date (julian day)
22°C
25°C
28°C

Figure 7

A.


Inoculation $F_{1,70}$ = 7.88 , P = 0.0065
Inoculation date $F_{1,70}$= 43.51 , P < 0.0001
Inoculation*Inoculation date $F_{1,70}$= 7.97 , P = 0.0062
Larval period (days)
Inoculation date (julian day)
Inoculated
Control

B.

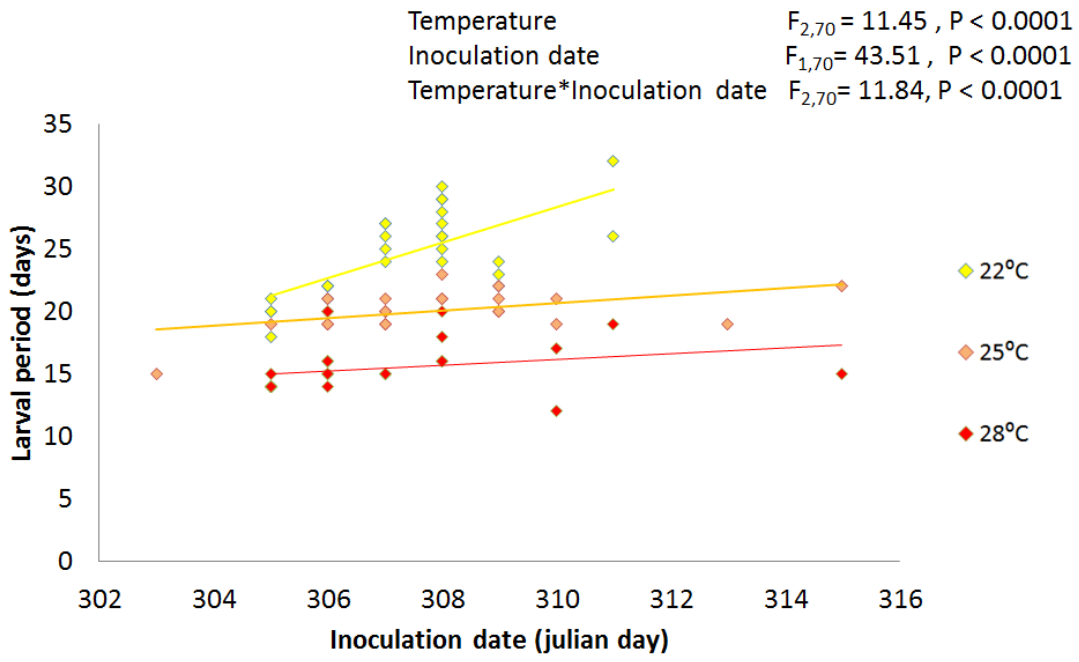

Temperature $F_{2,70}$ = 11.45 , P < 0.0001
Inoculation date $F_{1,70}$= 43.51 , P < 0.0001
Temperature*Inoculation date $F_{2,70}$= 11.84, P < 0.0001
Larval period (days)
Inoculation date (julian day)
22°C
25°C
28°C

Figure 8

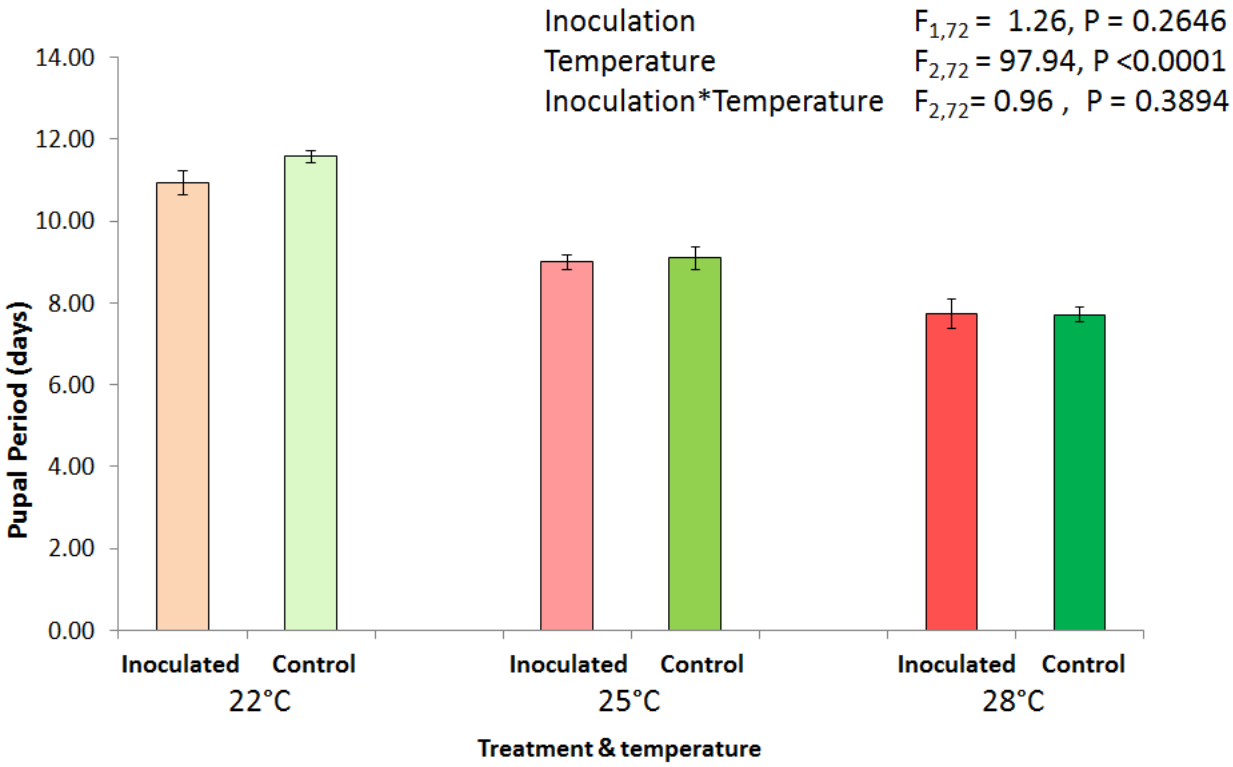

Inoculation $F_{1,72}$ = 1.26, P = 0.2646
Temperature $F_{2,72}$ = 97.94, P <0.0001
Inoculation*Temperature $F_{2,72}$= 0.96 , P = 0.3894
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
Pupal Period (days)
Inoculated
Control
22°C
Inoculated
Control
25°C
Inoculated
Control
28°C
Treatment & temperature

Figure 9

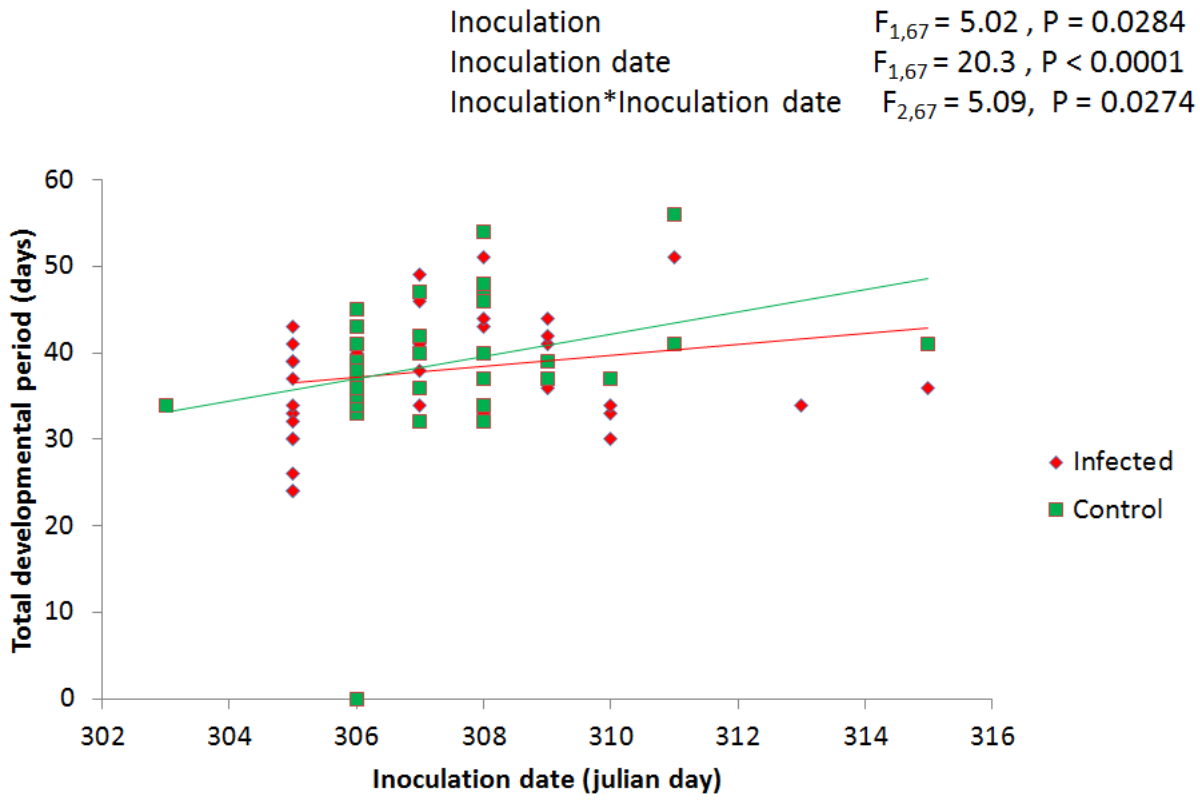

Inoculation $F_{1,67} = 5.02$, P = 0.0284
Inoculation date $F_{1,67} = 20.3$, P < 0.0001
Inoculation*Inoculation date $F_{2,67} = 5.09$, P = 0.0274
Total developmental period (days)
0
10
20
30
40
50
60
302
304
306
308
310
312
314
316
Inoculation date (julian day)
Infected
Control

Figure 10

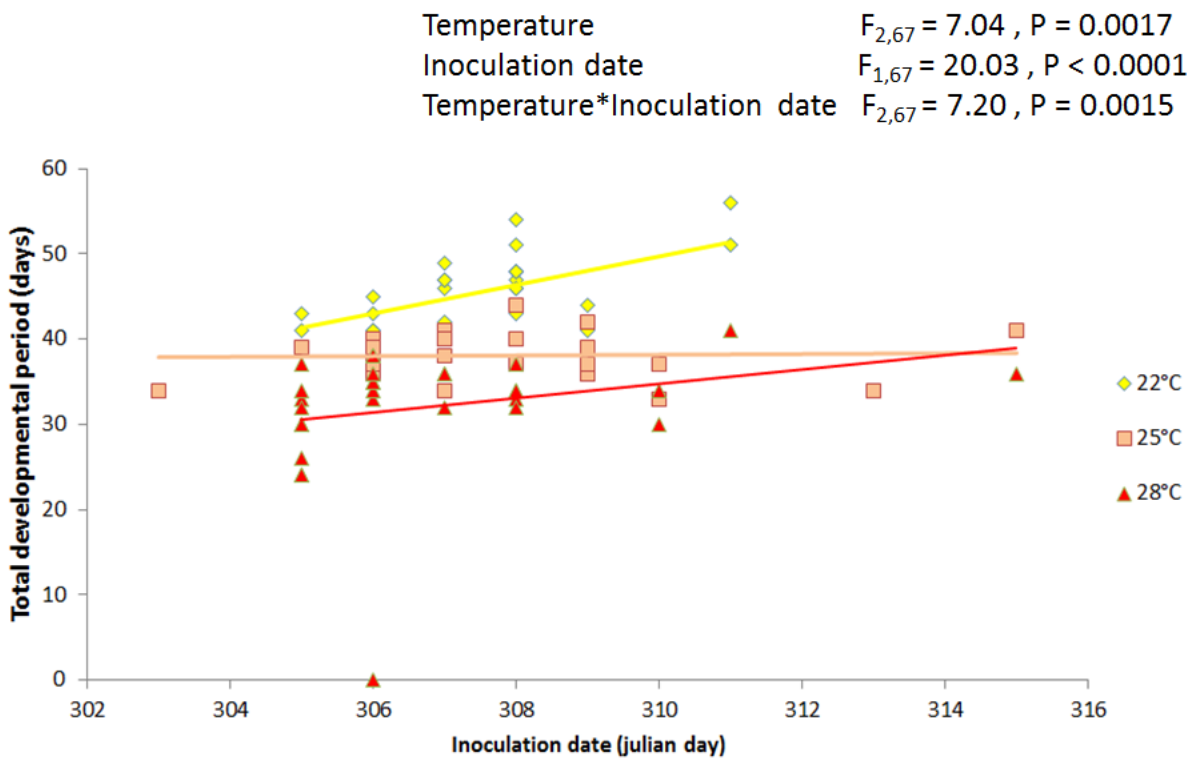

Temperature $F_{2,67} = 7.04$, P = 0.0017
Inoculation date $F_{1,67} = 20.03$, P < 0.0001
Temperature*Inoculation date $F_{2,67} = 7.20$, P = 0.0015
Total developmental period (days)
0
10
20
30
40
50
60
302
304
306
308
310
312
314
316
Inoculation date (julian day)
22°C
25°C
28°C

Figure 11

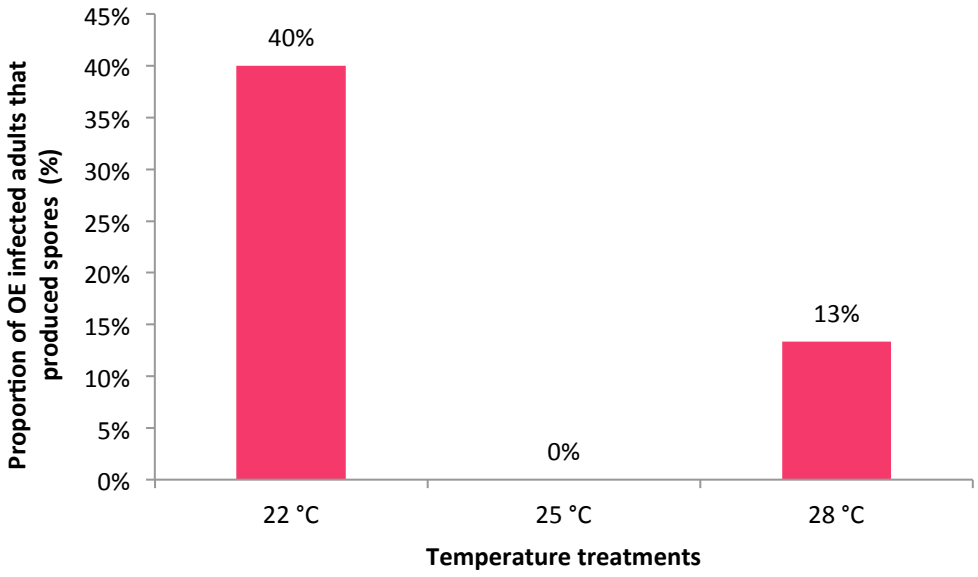
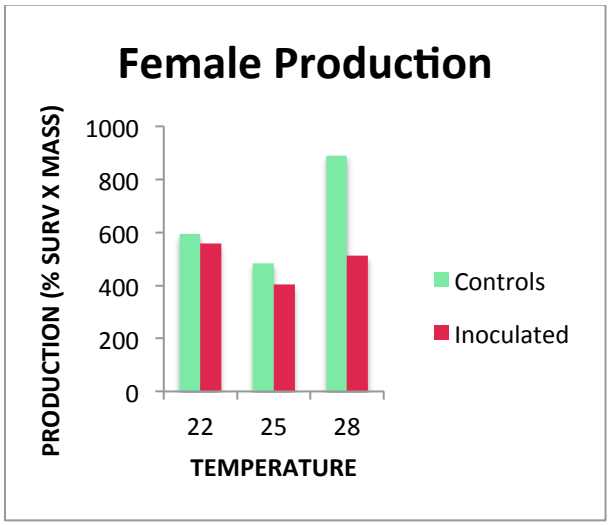
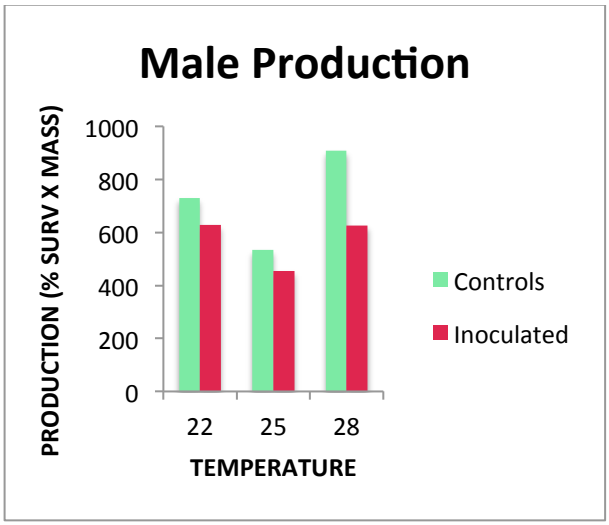

45%
40%
35%
30%
25%
20%
15%
10%
5%
0%
Proportion of OE infected adults that produced spores (%)
40%
0%
13%
22 °C
25 °C
28 °C
Temperature treatments

Figure 12

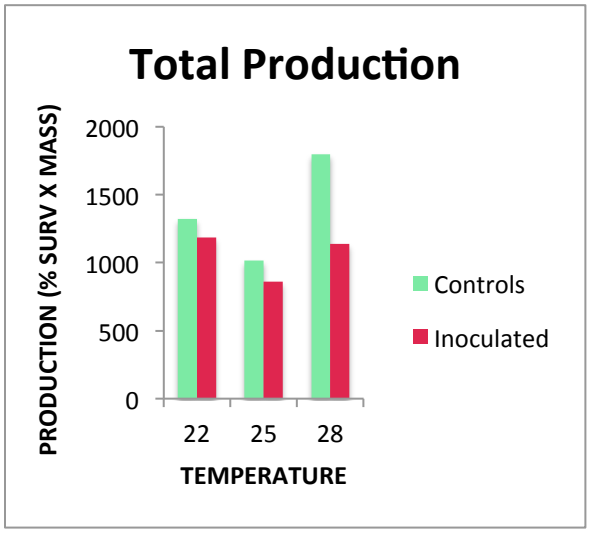
A



B



C



D

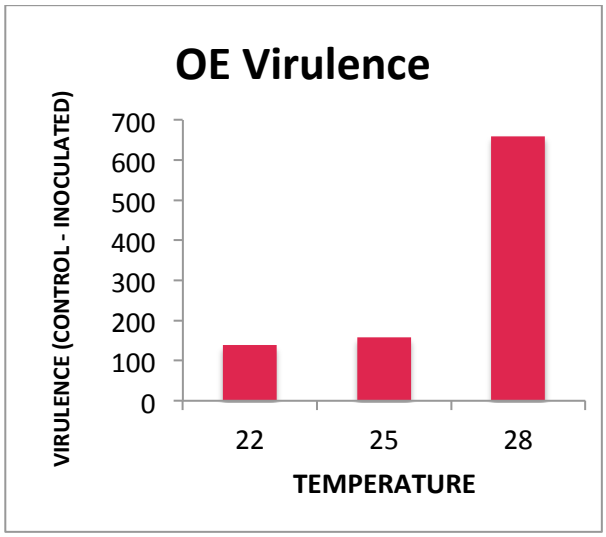


Table 1. Monarch butterfly developmental periods, pupal mass, mortality, and adult spore production. Monarchs were reared under three different experimental temperatures and larvae were either inoculated or not inoculated with OE parasites (control). The numbers in each cell represent the mean, the standard error (in parenthesis) and the number of butterflies used (sample size, n), respectively. The "mortality" row shows the proportion of individuals that died in each treatment. The last row, "spore production", shows the proportion of OE-inoculated butterflies that produced OE spores.

	22°C		25°C		28°C	
	Inoculated	**Control**	**Inoculated**	**Control**	**Inoculated**	**Control**
Larval period	23.93 (0.92) 15	25.58 (0.90) 12	19.83 (0.27) 12	20.30 (0.70) 10	14.80 (0.47) 15	16.14 (0.46) 14
Pupal period	10.93 (0.30) 15	11.58 (0.15) 12	9 (0.17) 12	9.10 (0.28) 10	7.73 (0.36) 15	7.71 (0.17) 14
Adult lifespan	9.20 (0.67) 15	9.83 (0.71) 12	9.25 (0.84) 12	8.70 (0.68) 10	9.27 (0.94) 15	11.15 (0.72) 13
Total development	44.07 (1.09) 15	47.00 (1.27) 12	38.08 (1.01) 12	38.10 (0.66) 10	31.80 (1.15) 15	35.15 (0.70) 13
Pupal mass	1177.13 (38.22) 15	1140.23 (43.25) 12	1253.51 (63.12) 12	1135.84 (40.36) 10	1144.15 (38.85) 15	1249.72 (38.13) 14
Mortality	50.00%	40.00%	62.50%	54.55%	51.61%	26.32%
Spore production	40%	N/A	0%	N/A	13%	N/A

Table 2. Summary of co-infection and temperature effect on OE virulence and monarch performance. The “Results” column indicates what happens when monarchs are experiencing the effect of (A) co-infection, and (B) an elevated temperature due to global warming. The “Implication” column describes what the results means in terms of a decrease or increase in OE virulence or monarch performance.

A

Co-infection effects	
Result	**Implication**
Inoculated larva dies	Increase in OE virulence in larvae
Larva develops faster	Decrease in larval exposure to predation
	Decrease in larval feeding time
Pupa weights less	Increase in OE virulence in pupae
Adult doesn't produce OE spores	Decrease in OE virulence in adult
	Decrease in OE transmission potential in adult

B

Elevated temperature effects	
Result	**Implication**
Larva develops faster	Decrease likehood of becoming co-infected
Pupa weights more	Increase in adult reproductive success (fitness)
Pupa develops faster	Decrease likelihood of pupal predation