Burn the Worm: Advancing *Manduca Sexta* as a New Model for Skin Injury and Infection

by Davita Wrone
Princeton High School, Princeton, New Jersey
Mentors: Mark Eastburn, Jennifer Smolyn, and Murad Alam

Abstract

Systemic infections after burn injury cause over 3,000 deaths in the United States each year. Developing an *in-vivo* model to improve treatment outcomes, however, frequently runs into ethical challenges due to the ongoing obstacle of the use of vertebrates in dermatological research. Fortunately, insects present advantages over vertebrate models due to their high reproductive rates, ease of care, and lack of ethical constraints; it would therefore be ideal to develop an invertebrate model for skin treatments that could be used to examine novel therapies. Experimental tests suggest that the tobacco hornworm, *Manduca sexta*, is an ideal candidate due to its large size and ability to withstand temperatures of 37ºC (human body temperature) for extended periods of time. When hornworms were burned three times and treated with one of four skin treatments, these larvae demonstrated a 4% survival rate after treatment with coal tar, a 33% survival rate when treated with cortisone, while honey allowed for an 84% survival rate and triple antibiotic ointment increased survival to 71%. The hypothesis that tobacco hornworms respond to skin treatments with outcomes that resemble vertebrates was sustained, suggesting a new method to test skin care products in a more ethical manner.

Introduction

Of the 137,061 burn victims between 2002 and 2011 in the United States, 3.3% died of their injuries, and among the most common complications were opportunistic bacterial infections (Zavlin et al., 2018). In other developed nations, the percentages can be even higher, such as 6.9% in the Netherlands (Bloemsma et al., 2008). Even more catastrophic is the fact that these numbers are surely even more catastrophic in the developing world, where such statistics are not as easily gathered, and people still use open flame to cook their food and heat their houses.

Invasive infection and sepsis in burns is the primary cause of death after burn injury, being the cause of 51% of deaths. *Staphylococcus aureus* is the main cause of infection, as a widespread use of antibiotics in recent years has made strains of antibiotic-resistant *S. aureus* (Williams et et al., 2009). *Pseudomonas aeruginosa* and *Eschericia coli* are two other major offenders.

Bacteria that cause such infections are highly prevalent in the environment, the vast majority belong to species such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Eschericia coli* (Merchant et al., 2015). Burns can be among the most complex injuries to treat because of the combination of the damage to the skin and the infection that can infiltrate the broken layers (Rowan et al., 2015), so further examination of best methods for mitigation and healing must be conducted on a wide scale before new or improved treatments reach patients. Studies that involve vertebrate organisms such as mice and pigs generally require ethical review, and many treatments that might open new avenues of research are rejected because of the physical harm, pain, and suffering that they may cause (Maslova et al., 2020).

At present, studies indicate that insects exhibit nociception (avoidance of harmful stimuli), but their responses cannot be considered the vertebrate equivalent of "pain," which requires more complex neural comprehension (Burell, 2017). It is therefore suggested that experiments that cause injury to insects would not be considered unethical (Maslova et al. 2020).

In 2020, an article was released in the journal *Frontiers in Microbiology* that proposed a new invertebrate model for burn research–larvae of the greater wax moth, *Galleria mellonella* (Maslova et al., 2020). Other investigations have proposed the silkworm, *Bombyx mori*, for a variety of medical analyses, including toxicology (Abdelli et al., 2018), oncology (Meng et al., 2017), immunology (Hamamoto et al., 2012), and gerontology (Kunugi & Ali, 2019). Silkworms have even been investigated for antiviral proteins (Lü et al., 2018) and their entire genome has been sequenced (Kawamoto et al., 2019) to advance studies of protein formation (Zhou et al. 2008) and genetic engineering (Zabelina et al. 2015) for medical (Xu, 2014) and industrial applications (Teramoto et al., 2018).

Flaws in the two aforementioned invertebrate models are clear: waxworms are relatively small and pose challenges in localizing specific injuries. Additionally, silkworms are best kept at room temperature (Li et. al, 2020). Limited experience with both waxworms and silkworms also demonstrate that there are many challenges when it comes to keeping and breeding these organisms (personal observation). For these reasons, it has become necessary to seek a new model–which has both a larger size and higher temperature tolerance–for further treatments.

The Carolina hawkmoth, *Manduca sexta*, is widely used in scientific research while in larval form, called the tobacco hornworm. Like the silkworm, its genome has been sequenced (Kanost & Blissard, 2015), and it has been used in studies as diverse as neuroscience (Caron et.

al, 2020) and fungal infection (Lyons et al., 2020). In addition to its large size and extreme ease of care (personal observation, as their feeding and cleaning regiments were simple), preliminary trials demonstrate that these larvae can be incubated at 37ºC for at least three days continuously without increased mortality (personal observation.) This project aims to determine whether skincare products such as coal tar will cause abnormal cell growth in a similar organism, the tobacco hornworm (*Manduca sexta*), which has important implications for the use of such compounds in humans. Among the many advantages of this insect are a well-characterized genome (Gershman et al., 2021) and an ability to tolerate human body temperatures (personal observation, as they survived in pre-trials the same amount in and out of the incubator). In addition, the purported healing benefits of other treatments will be tested, in order to determine if they are able to promote healing (tested by survivability.)

The testable questions to be investigated are as follows: “Can treatments that are suspected to cause skin cancer in vertebrates be replicated in an invertebrate model?”, and “Do certain compounds help to reverse skin damage and promote healing?” The hypothesis that guides this experiment states that if hornworms are exposed to ultraviolet radiation and/or coal tar, then they will exhibit uncontrolled cell growth in the same manner as cancerous cells because these insects exemplify many of the same genetic regulatory mechanisms that are found in vertebrates.

This experiment has many real-world implications. First, is using a non-mammalian model for burn injury and treatment to help learn more about it. *Manduca Sexta* are cheap (as is their food), small, and easy to care for. Establishing them as a workable model for burn injury and treatment could increase studies, knowledge, and research on how to treat burns and prevent subsequent infections.

One possibility for treatment is honey. Honey is thought to have antimicrobial properties, although the mechanism by which it works is not well explored (Mandal & Mandal, 2011). Honey is easily accessible to many, and, if proven to work as treatment for burns, could help many people, especially those in third-world countries who might not have access to other ointments and creams. Finally, although not tested so far in this project, the possibility for learning more about skin cancer, its causes, and possible cures. Skin cancer is the most common cancer in the United States and it causes over 7,000 deaths every year (American Academy of Dermatology). Learning as much as possible about how to prevent it is essential.

Materials and Methods:

Tobacco hornworms (*Manduca sexta*) were bred from adult Carolina hawkmoths, and raised on a commercial feed that was purchased from Oregon Silkworms, LLC. Through the first four instars, caterpillars were kept at room temperature and fed a steady diet of prepared food. A total of 180 healthy hornworms were raised.

After their emergence as fifth instars, at which point tobacco hornworms are at their largest, three burns were administered on the left side of each larva with a 60-watt soldering iron (LDK brand), between the segments where the second and third abdominal prolegs are located, third and fourth abdominal prolegs, and fourth and fifth abdominal prolegs are located (see Figure 3). These locations were chosen because they were not close to the head nor heart (which are located near the front and top, respectively); care was also taken to avoid the spiracles, which is how insects breathe.

Upon receiving the three burns, which were timed at one second each, a sterile swab was used to administer one of four treatments: coal tar (negative control, considered toxic), cortisone (anti-inflammatory, but not anti-bacterial), honey (suspected to have antimicrobial properties, as suggested in the Netflix series *Anne with an E*), and triple-antibiotic ointment (positive control) (Figure 4). In each test group, a total of 45 hornworms were used.

For incubation at 37ºC, individual hornworms were placed in a clear, plastic tube (repurposed fruit fly culture tubes) with approximately 10 grams of food, capped with a foam plug (see Figure 5), and placed in a Happybuy 25 L incubator for three days. Caterpillars were checked each day and supplied with fresh food when necessary, as the fifth larval stage is a time of optimal eating and growth. Dead hornworms were removed as soon as melanization (darkening of the body) was apparent. After the three days, hornworms were returned to room temperature and allowed to eat until reaching pupation, at which point they were buried in moistened aspen wood shavings and kept in a 6 L Sterilite bin until emerging as adults. Once hawk moths shed their pupal husks and assumed their adult form, all were visually assessed for any abnormalities. This was done by comparing multiple control hawkmoths and some treated with coal tar to check for any vivid abnormalities (enlarged body parts, seemingly underdeveloped ones.)

After the three-day incubation, every hornworm in each survival group was assigned a number: 0 for dead, and 1 for alive. These data were then entered into a table for analysis with One-Way Analysis of Variance (ANOVA) and post-hoc Tukey Honestly Significant Difference (HSD) tests in order to determine statistical significance.

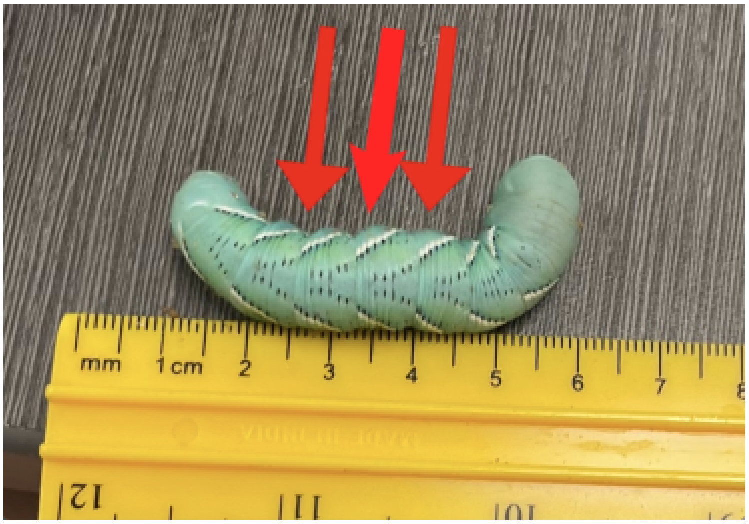

Figure 1: The third and fourth abdominal prolegs of the hornworms (*Manduca sexta)*, as shown by the red arrows. These are the spots at which the hornworms were burned with the soldering iron. Care was taken to avoid the head (far left), the heart (top), and the spiracles (yellow/black dots), which is how hornworms breathe.

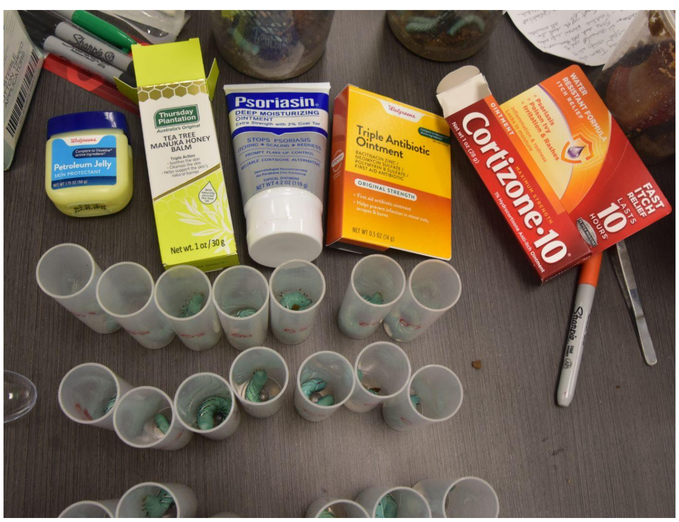

Figure 2: The hornworms (*Manduca sexta*) in the plastic tubes after being burned and treated with: coal tar, cortisone, honey, and triple-antibiotic ointment (positive control). Note: this picture was taken in an early trial where tea tree balm and vaseline were being tested instead of honey.

Figure 3: Containers in the incubator with the *Manduca sexta* fifth instars at 37^{o}C.

<u>Results</u>

As predicted, significant differences were found between those treatments that contained antimicrobial compounds and those that did not; survival was much higher for those hornworms that were treated with honey or triple-antibiotic ointment (Figure 6). Coal tar and cortisone had lower survival rates when compared with triple-antibiotic ointment, as confirmed by One-Way ANOVA [$F(3,229) = 50.3812$, $p < 0.0001$]. Post-hoc comparisons using the Tukey HSD test determined significant differences between the survival rates of hornworms treated with coal tar vs. cortisone (Q statistic = 5.7761, $p < 0.01$), cortisone vs. honey (Q statistic = 9.8534, $p < 0.01$), and both coal tar vs. triple-antibiotic ointment (Q statistic = 13.0674, $p <0.01$), and cortisone vs. triple antibiotic ointment (Q statistic = 7.2662, $p <0.01$). The only treatment for which there was no significant difference was honey vs. triple-antibiotic ointment (Q statistic = 2.6299, $p = 0.2486$).

With that statistical test, the cortisone and coal tar were found to be statistically insignificant from each other, as well as the honey and triple antibiotic ointment.

All surviving larvae that reached the pupal stage emerged without any observed abnormalities, and were able to survive long enough to mate and lay eggs. Therefore no statistical analysis was performed in the outcome of metamorphosis between treatments.

Figure 6: Survival of hornworms after three burns in the fifth instar stage and treatment with one of four skin preparations. Larvae in groups (*n* = 57 for each) were tracked from burning and incubation at 37ºC through metamorphosis into the adult Carolina hawk moth. Groups that are not statistically significant from each other are the triple antibiotic ointment and honey & the cortisone and coal tar.

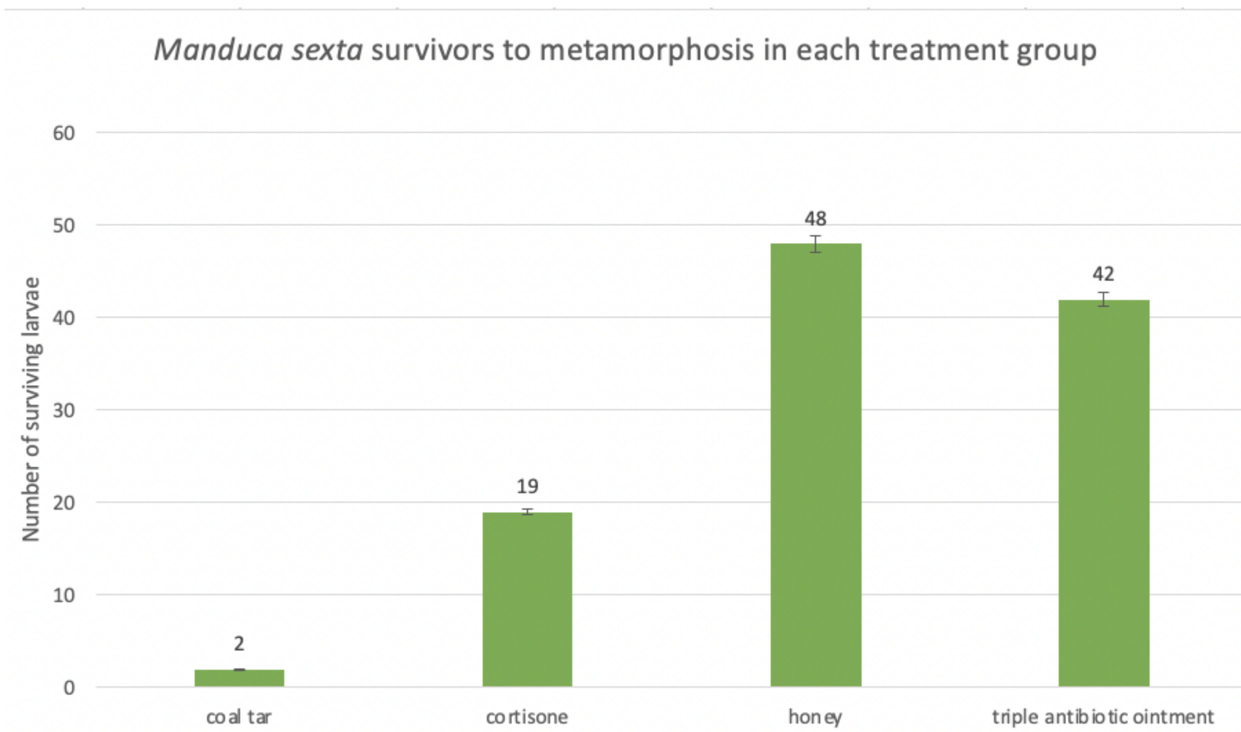

Figure 6: Survival of hornworms after two burns in the fifth instar stage and treatment with one of four skin preparations. Larvae in groups (*n* = 57 for each) were tracked from burning and incubation at 37ºC through metamorphosis into the adult Carolina hawkmoth. Coal tar and cortisone are not statistically different from each other and honey and triple-antibiotic ointment are not statistically significant from each other.

Discussion

While the results generally confirm the original hypothesis, a few of the outcomes were surprising. Coal tar has been suspected of containing genotoxic and potentially carcinogenic compounds (Borská et al., 2004), yet epidemiological studies have failed to produce any appreciable links between coal tar treatment for skin conditions and cancer (Roelofsen et al., 2010). At the same time, coal tar has been implicated in immunosuppression when used in psoriasis treatments in humans (Borska et al., 2008), which could potentially suggest that application of coal tar may have inhibited the treated hornworms' ability to fight off opportunistic bacterial infection. This may be an explanation for why the coal tar experimental group had such a high mortality rate (96%), though more investigation is needed.

While invertebrates generally have an innate immune system, and vertebrates have both an adaptive *and* an innate immune system, certain components of the insect immune response (including inflammation) parallel those of vertebrates (Hillyer, 2016). This may explain why hornworms treated with cortisone also had a high mortality rate (33% mortality), because cortisone is known to have anti-inflammatory (and presumably anti-immune) effects (Barnes, 2006). No differences in metamorphosis were observed, implying that cortisone does not have any mutagenic effects.

The most surprising finding from this study was the high survival rate of hornworms that were treated with honey (84%), which was determined to be statistically insignificant from the survival rate of those hornworms that were treated with antibiotic ointment (71%). Upon further research into the scientific literature, honey does appear to have antibiotic properties, in part because of its ability to produce hydrogen peroxide, although non-peroxide honey does also appear to be effective (Mandal & Mandal, 2011). In addition to the potential for honey to actively kill microorganisms, it also helps aid in wound healing by keeping the wound moist and providing a protective barrier from environmental contamination (Mandal & Mandal, 2011). As with the other treatments, further investigation is warranted, particularly if honey has any effect on the inflammatory response or hemocyte activity (hemocytes are the "germ-killer" cells within insects). Comparisons should also be made with the triple-antibiotic ointment, because antibiotic resistance continues to be a problem in many healthcare settings (Aslam et al., 2018). Wound care that could use honey to prevent infection would have important implications for the future of antibiotic resistance, as well as healthcare treatments in the developing world.

In the future, further investigation of coal tar and why it seems to harm the *Manduca Sexta* should be studied. Coal tar is thought to have possible cancerous properties (Aben 2007). In the future, doing more coal tar treatments and sequencing the genome of the "treated" *Manduca sexta* could provide insight into the effects of coal tar. To contrast, further studies into the ways in which honey seems to help the *Manduca sexta* should be studied, for reasons already stated in the introduction. The mechanisms by which both of these substances work to improve or harm organisms is not known, but could have major implications in the field of medicine.

In addition, another possibility for expansion on this project is to culture bacteria samples from the burned worm. This way, it could be tested what bacteria actually invaded the worm's system and caused them to do so that better, more targeted treatments could be created for them.

Overall Conclusions

The hypothesis that fifth instar larvae of *Manduca sexta* could be burned, treated with over-the-counter ointments, incubated at 37ºC, and recapitulate human burn/infection models was sustained. More evidence is required to determine how, exactly, honey is able to prevent infection, as well as the effects of both coal tar and cortisone on the insect immune system in comparison with human experiments. The significance of this project is the demonstration of a potentially valuable *in vivo* model of burn injury and opportunistic bacterial infection that has implications for both dermatology and healthcare as a whole.

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