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OF HORSENETTLE & HORNWORMS: THE DAMAGE MECHANISMS OF NON-GLANDULAR TRICHOMES AND THEIR EFFECTS ON HERBIVORES

A Dissertation in

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ABSTRACT

In their endless struggle to survive, organisms constantly suffer damage from their surrounding environment. Pathogens infect. Toxins poison. Predators bite. Based on their own struggle for survival, plants have evolved numerous mechanisms to defend themselves against herbivory. These mechanisms are divided into two, broad categories: chemical and physical defenses. Chemical defenses include distasteful and toxic compounds. Physical defenses include spines, leaf toughness, and trichomes that make it difficult for herbivores to consume plants. Trichomes are particularly diverse, grow in many forms, and may or may not contain a distal gland. While glandular trichomes have been shown to defend plants by trapping or poisoning small herbivores, non-glandular trichomes are less well-studied.

This dissertation explored the short-term, long-term, and transgenerational effects experienced by a lepidopteran herbivore after consuming non-glandular trichomes. To examine the effects of trichomes alone, I removed the trichomes from Horsenettle (*Solanum carolinense*) and added them to an artificial diet that lacked other leaf chemicals or structures. To examine the effects of consuming trichomes *in situ* on leaves, I created two treatments, one with trichomes and one with trichomes removed, using the same species to maintain consistent leaf chemistry. I observed the effects on Tobacco Hornworms (*Manduca sexta*) after consuming these diets by measuring their growth, efficiency, and survival. I collected eggs of this first generation to explore potential, trichome-induce transgenerational effects on offspring growth, efficiency, and survival. Finally, I sought to understand which characteristics of non-glandular trichomes damaged larvae.

Together, these studies revealed that non-glandular trichomes, consumed as part of a natural diet, chemically and physically damaged hornworms. The resulting damage to the midgut epithelium allowed the gut contents to leak into the surrounding hemocoel which lead to energetic

resources being diverted from growth to other metabolic processes; likely tissue repair and immune response mechanisms. As a result, larvae ate less diet and gained less mass. Their offspring also diverted energy to other metabolic processes, were smaller, and more likely to survive. Ultimately, this collection of work not only provides a detailed example of the long-term and transgenerational effects of damage, but also identified the mechanism behind the damage.

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EPIGRAPH

To a Mouse, on Turning Her Up in Her Nest With the Plough, November, 1785

Wee, sleekit, cowrin, tim'rous beastie,
O, what a pannic's in thy breastie!
Thou need na start awa sae hasty,
Wi' bickering brattle!
I wad be laith to rin an' chase thee,
Wi' murd'ring pattle!

I'm truly sorry man's dominion,
Has broken nature's social union,
An' justifies that ill opinion,
Which makes thee startle
At me, thy poor, earth-born companion,
An' fellow-mortal!

I doubt na, whiles, but thou may thieve;
What then? poor beastie, thou maun live!
A daimen icker in a thrave
'S a sma' request;
I'll get a blessin wi' the lave,
An' never miss't!

Thy wee bit housie, too, in ruin!
It's silly wa's the win's are strewin!
An' naething, now, to big a new ane,
O' foggage green!
An' bleak December's winds ensuin,
Baith snell an' keen!

Thou saw the fields laid bare an' waste,
An' weary winter comin fast,
An' cozie here, beneath the blast,
Thou thought to dwell-
Till crash! the cruel coulter past
Out thro' thy cell.

Thy wee bit heap o' leaves an' stibble,
Has cost thee mony a weary nibble!
Now thou's turn'd out, for a' thy trouble,
But house or hald,
To thole the winter's sleety dribble,
An' cranreuch cauld!

But, Mousie, thou art no thy-lane,
In proving foresight may be vain;
The best-laid schemes o' mice an' men
Gang aft agley,
An' lea'e us nought but grief an' pain,
For promis'd joy!

Still thou art blest, compar'd wi' me
The present only toucheth thee:
But, Och! I backward cast my e'e.
On prospects drear!
An' forward, tho' I canna see,
I guess an' fear!

– Robert Burns

Chapter 1

Introduction

Plants have evolved extensive suites of chemical and physical defenses to protect themselves against herbivory. Chemical defenses are well studied and include protective secondary metabolites such as antibiotic compounds to protect against microbes, alkaloids that make tissues unpalatable, toxins that can poison herbivores, and volatile compounds that can recruit tertiary predators to remove small herbivores from the plant (Fraenkel 1959, Cipollini and Levey 1997, Wittstock and Gershenzon 2002, Halitschke et al. 2007). Herbivores that consume chemical plant defenses show diminished growth, survivorship, and reproduction (Fox et al. 1995, Pechan et al. 2002, van Asch et al. 2007, 2010, Raguso et al. 2007, Gog et al. 2014, Portman et al. 2015a, Veyrat et al. 2016). In some cases, this is due to chemical burns blistering the midgut epithelium of herbivores, which leads to the upregulation of energetically expensive tissue repair (Pechan et al. 2002, Barbeta et al. 2008, Fescemyer et al. 2013). Plant chemical defenses have also been shown to elicit transgenerational effects. Offspring of parents exposed to plant chemical defenses tend to be larger, develop more quickly, and are more likely to survive than offspring on non-exposed parents (Rossiter 1991a, 1991b, Fox et al. 1995, Awmack and Leather 2002, van Asch et al. 2007).

Plant physical defenses also inhibit herbivore feeding and include methods such as spines, toughness, and trichomes. Plants that produce sharp spines are able deter large herbivores such as cattle, goats, giraffes, and kangaroos by injuring them as they bite stems and leaves (Bassett and Munro 1986, Milewski et al. 1991, Belovsky et al. 1991, Gowda 1996, Obeso 1997, Hanley et al. 2007). However, spines tend not to deter small herbivores such as insects that can fit between spines, eat around spines, or even reside inside of large spines (Janzen 1966, Medeiros

and Moreira 2005, Serpi unpublished data). The stiff structure of spines is the result of cellulose which makes spines difficult to digest (Belovsky et al. 1991). Cellulose also toughens leaves and stems making them more difficult for both large and small herbivores to bite or pierce plant tissues (Rossiter 1991a, 1991b, Fox et al. 1995, Awmack and Leather 2002, van Asch et al. 2007). Even if herbivores manage to bite, the additional cellulose can reduce the nutritional density (Rodriguez et al. 1984). Herbivores that consume low nutritional dense diets tend to be smaller and less likely to thrive (Moran and Hamilton 1980, van Asch et al. 2010, Triggs and Knell 2012a). Nutrition quality in the parental generation can have transgenerational consequences such as producing fewer offspring that are smaller, with reduced immune reactivity, and are less likely to survive (van Asch et al. 2007, 2010, Triggs and Knell 2012b).

Additionally, plants produce small epidermal hairs called trichomes (Levin 1973). Some trichomes, glandular trichomes, defend plants by producing a sticky, distal gland that physically traps small herbivores or glue shut the mandibles of chewing insects (Gibson 1971, Kennedy 2003). This inhibits the herbivores ability to feed on plants and results in starvation, reduced growth, and delayed development (Gibson 1971, Kennedy 2003). Herbivores that manage to consume glandular trichomes may be poisoned by the toxic chemicals produced within the gland which leads to the same effects as when consuming other leaf chemical defenses (Thurston 1970, Kennedy 2003). Glandular trichomes have also been shown to defend plants by producing volatile chemicals that attract predators of herbivores (Kennedy 2003) and by detecting the presence of herbivores which induces additional defenses (Tooker et al. 2010). Other trichomes do not produce a distal gland. Rather than plant defense, these non-glandular trichomes are typically associated with water retention, respiration, and photosynthesis (Brewer et al. 1991, Konrad et al. 2015). Some studies, however, have linked non-glandular trichomes to slowing the movement of insects, hampering insects' ability to quickly bite leaves, and reducing insect oviposition (Johnson 1953, Wellso 1973, Pillemer and Tingey 1976, Eisner et al. 1998, Medeiros

and Moreira 2002, Mitchell et al. 2016, Kariyat et al. 2017). However, there is reason to believe that these seemingly benign hairs also play a destructive role in plant defense after being consumed by an herbivore.

Non-glandular trichome density increases as part of a plant's induced response following herbivore damage (Agrawal 1999, Kariyat et al. 2013, Pashalidou et al. 2015). It is possible that this is incidental, but considering the resource allocation for trichome growth, non-glandular trichomes may confer a benefit for plants to resist subsequent attack. Secondly, intact non-glandular trichomes have been recovered within the frass (fecal matter) of insects following leaf consumption (Wellso 1973, Kariyat et al. 2017). Due to the relative scale, the stiff points of undigested trichomes likely pose a physical threat to the internal tissues of insect larvae. Observations have revealed that trichomes consumed by larvae can catch on the peritrophic membrane within the larval gut (Kariyat et al. 2017) and, as observed in a single larva, possibly pierce the midgut epithelium (Wellso 1973). It is unclear if the dissection process affected the positioning of the trichomes, but if non-glandular trichomes are able to pierce through the peritrophic membrane *in vivo* and damage the surrounding midgut epithelium, it may lead to similar effects of damage to the midgut epithelium by chemical plant defenses (Pechan et al. 2002, Barbeta et al. 2008, Fescemyer et al. 2013).

With this in mind, I hypothesized that larvae reared on diets containing non-glandular trichomes would suffer internal damage. Subsequent tissue repair and possible immune response mechanisms would divert energy resources from growth to reproduction (Freitak et al. 2003, Boggs 2009). Resource limitations must balance these costs by diverting the energy for these process from energy resources normally allocated for routine processes like growth and reproduction (Herms and Mattson 1992, Rauw 2012, Kariyat et al. 2013). As a result, organisms that survive after suffering bodily harm tend to be smaller and produce smaller or fewer offspring than their unharmed peers (Martin et al. 1977, Fox et al. 1995, Fescemyer et al. 2013). The effects

of damage and resource limitation may extend beyond the life history of an organism and influence their offspring (Roach and Wulff 1987, Mousseau and Dingle 1991, Rossiter 1996, Mousseau 1998). Such transgenerational effects have been found in a wide range of species including plants (Agrawal et al. 1999, Nihranz and Stephenson 2014), insects (Fox et al. 1995, Rossiter 1996, Freitak et al. 2014), crustaceans (Agrawal et al. 1999), birds (Whittingham et al. 2002), reptiles (McCormick et al. 2017, Owen et al. 2018, Ensminger et al. 2018), and mammals (Catalano 2003, Lumey et al. 2011). Studies of transgenerational effects in Lepidoptera often only examine the initial population effects (Woestmann and Saastamoinen 2016), but there is some indication that transgenerational effects can last even after offspring metamorphosis (Cahenzli and Erhardt 2013).

Previous studies have attempted to measure the effects experienced by herbivores after consuming non-glandular trichomes, but fell short in one of two ways. First, some studies were only loosely controlled and did not sufficiently isolate the effects of trichomes from the nutritional and defensive chemistry of the leaves. This included comparing different species, difficult cultivars, or different breeding histories (Gibson 1971, Maxell and Jennings 1980, Khan et al. 1986, Haddad and Hicks 2000, Kariyat et al. 2017). While trichome density changed in each of these scenarios, it is also likely the chemical content and concentration also varied between treatments. In some cases, the chemical differences between treatments were even acknowledged or previously measured (Khan et al. 1986, Haddad and Hicks 2000, Portman et al. 2015b).

Secondly, in studies that controlled both trichome density and chemical content, researchers often only examined the ability of trichomes to inhibit biting, but did not follow the effects of non-glandular trichomes after consumption by an herbivore. One common metric was a larval choice test between treatments with and without trichomes which tests an herbivores preference, but does not indicate if trichomes harm herbivores (Agrawal 1999, Fordyce and Agrawal 2001, Medeiros and Moreira 2005, Kariyat et al. 2017). In nature, most larvae rarely

have the opportunity to choose their diet and simply consume the plant host upon which its mother has oviposited. When larvae do choose a host, their choice is usually limited to conspecifics or related species that also produce trichomes. For example, tobacco hornworms (*Manduca sexta*) are oligophagous specialist herbivores on solanaceous plants, most of which produce trichomes, including tobacco (*Nicotiana tabacum*), tomato (*Solanum lycopersicum*), deadly nightshade (*Atropa belladonna*), petunia (*Petunia* spp.), and horsenettle (*Solanum carolinense*) (Yamamoto and Fraenkel 1960, Wise 2007). Choosing a natural diet without trichomes may be virtually impossible and therefore the ability to choose a trichome-free diet may be irrelevant.

Studies that did examine the effects of non-glandular trichomes after consumption only monitored short-term responses and did not assess long-term development. Most commonly, larvae were monitored for weight gain over a 24 or 48 hour period (Agrawal 1999, Kariyat et al. 2017). The decline in weight gain among larvae reared on treatments containing trichomes could indicate that trichomes functioned like spines by making it difficult to bite leaves and larvae simply consumed less leaf tissue and the reduced weight gain is a result of insufficient nutrient acquisition. Alternatively, following consumption, trichomes could internally damage larvae, forcing resource allocation trade-offs that lead to long term consequences for herbivore survival and their offspring.

I have found only one study that controlled for both trichome presence and leaf chemistry examined the diet consumption and conversion efficiency across the life time of an herbivore. That study found that a specialist grasshopper species was unaffected by trichome consumption, except that grasshoppers reared on diet with trichomes were more likely to survive (Smith and Grodowitz 1983). A generalist grasshopper species reared on the same diet with trichomes consumed less diet, gain less mass, had greater assimilation and lower conversion efficiency, and were less likely to survive as compared to grasshoppers reared on diets in which the trichomes

had been removed using watchmaker's forceps (Smith and Grodowitz 1983). These results seem to indicate that specialist herbivores are equipped to consume trichomes, whereas generalist herbivores experience the same resource diversion from growth to other metabolic process as seen in studies of leaf chemical defenses. Another interesting note is that grasshoppers are hemimetabolous and the life-long effects of consuming trichomes are likely different for holometabolous insects, like Lepidoptera, which undergo very different biochemical processes during metamorphosis (Berenbaum and Isman 1989, Gullan and Cranston 2011).

To understand the effects of consuming non-glandular trichomes without the confounding effects of leaf chemistry, I have used a natural system and developed a set of treatments to isolate the effects of trichomes. Horsenettle (*Solanum carolinense*) is an endemic host of tobacco hornworms (*Manduca sexta*) and produces non-glandular, stellate trichomes (further details for each species are included at the end of this chapter to reduce redundancy in each subsequent chapter). To avoid confounding effects of nutritional and defensive leaf chemistry, I created two treatments using horsenettle leaves. In one treatment, I shaved the leaves to remove the trichomes but left other leaf architecture and chemistry intact, and in the other treatment I left the trichomes *in situ* on the abaxial and adaxial leaf surfaces. Taking this a step further, for a third and fourth treatment, I added trichomes to an artificial diet and used artificial diet without trichomes to remove the effects of leaf chemistry.

Using these treatments, I monitored the long-term effects of consuming trichomes by measuring the survival, development time, and reproduction of hornworms. I also measured their efficiency in converting diet to body mass to find resource allocation trade-offs due to potential bodily damage (Chapter 2). After observing the consequences of consuming trichomes, I wanted to determine how trichomes damage larvae and whether the midgut is pierced. In the first experiment, I manipulated trichomes to isolate their nutritional, chemical, and physical characteristics. In the second, I used fluorescent powder to track the movement of diet within the

larvae to determine the extent of the damage and if the midgut epithelium was punctured in vivo (Chapter 3). Finally, I then followed the offspring of larvae reared on diets containing trichomes to see if trichome consumption lead to transgenerational effects. Again, I measured the survival, development time, and conversion efficiency of larvae (Chapter 4). A timeline showing the order and length of experiments can be found below (Fig. 1.1).

Together these chapters specifically target the effects of consuming non-glandular trichomes. Furthermore, they reveal the mechanism by which non-glandular trichomes internally damage herbivores. In doing so, this dissertation should illustrate the long-term and transgenerational consequences of damage by using an ethical and ecologically relevant source of damage.

Study System

Tobacco hornworms (*Manduca sexta*)

Manduca sexta (commonly Tobacco Hornworms or Tobacco Hawk Moths) is a member of the Sphingidae family and native to North America (Hodges 1983) (Fig. 1.2). They are considered oligophagous specialist herbivores of solanaceous plant hosts; including tobacco (*Nicotiana tabacum*), tomato (*Solanum lycopersicum*), deadly nightshade (*Atropa belladonna*), petunia (*Petunia* spp.), and horsenettle (*Solanum carolinense*) (Yamamoto and Fraenkel 1960, Wise 2007).

Each female moth produces an average of 400 eggs which are individually laid on these selected host species (Sasaki and Riddiford 1984). Smooth, green larvae with white, lateral stripes and red, dorsal horn hatch after 4-7 days (Hodges 1983). Following five consecutive instars, and gaining approximately 10 grams of body mass, larvae enter the prepupation wandering phase (Hodges 1983). Occasionally, larvae undergo an additional sixth instar if they are unable to gain sufficient weight due to a lack of resources or reduced dietary conversion efficiency (Kingsolver 2007). Tobacco hornworms pupate underground and undergo complete metamorphosis in about 40 days (Hodges 1983). Adult moths display brown, bark-patterned wings and six, yellow, lateral spots along the abdomen (von Linné 1763, Hodges 1983). Adults are nocturnal, nectar-feeders and live for 7-14 days (Hodges 1983). *M. sexta* can produce up to three generations each summer season (Kingsolver et al. 2012).

Manduca sexta have evolved several features to defend themselves from sources of environmental harm. Larvae camouflage themselves from predators by sequestering plant xanthophylls that provide their characteristic green color which matches their specific plant host (Dahlman 1969, Kawooya et al. 1985). As part of their solanaceous diet, larvae are exposed to

plant chemical defenses such as nicotine and other harsh alkaloids compounds (Fraenkel 1959, Cipollini and Levey 1997, Wittstock and Gershenzon 2002, Halitschke et al. 2007). However, *M. sexta* larvae have evolved to be able to digest up to 90% of these toxic compounds (Wink & Theile, 2002). As a result, the digestive tract of *M. sexta* larvae are highly alkaline with an average pH of 11 (Dow 1992), which renders their gut relatively inhospitable to microbial growth (Hammer et al. 2017). Larvae also have some protection from physical plant defense and other abrasive particles due to a chintinous lining around their foregut and hindgut (Gullan and Cranston 2011). However, the midgut is not protected by a layer of chitin due to the need for efficient nutrient absorption (Gullan and Cranston 2011). Here the food bolus is enveloped by a thin, flexible mesh of chitin called the peritrophic membrane which allows nutrients to flow through to the surrounding epithelium, while preventing large particles from reaching this delicate membrane (Gullan and Cranston 2011). If the epithelium is breached by foreign bodies or microbes enter the hemocoel, larvae have a melanizing encapsulation mechanism to encase the offending particles and can elicit an antimicrobial response (Kanost et al. 2004, Gullan and Cranston 2011).

Overall, *M. sexta* biology is fairly well studied and research on the species now includes a recently-published complete genome (Kanost et al. 2016). In addition to this, their large size and ability to be easily reared in a laboratory pushes them closer to achieving model organism status. As a result, *M. sexta* are used in a wide range of studies. This includes more traditional ecological work on topics such as tri-tropic interactions, parasitism, and plant defenses (Beckage and Kanost 1993, Halitschke et al. 2007, Tooker et al. 2010, Kariyat et al. 2013, Portman et al. 2015b), but also includes more medically focuses work such as studies on plague toxins and Alzheimer's disease (Montemayor et al. 1990, Hares et al. 2008). Therefore, further studies on their response to damage and artificial diets may be broadly applicable.

For this study, larvae for the experimental colony originated from with *M. sexta* eggs purchased from Great Lakes Hornworm (Romeo, MI) and annually supplemented with wild individuals gathered around Centre County, Pennsylvania. The colony was reared under summer-like conditions within the laboratory (16:8 hour light:dark photoperiod, 26 ˚C, ambient humidity). Rearing cups were assembled by pouring prepared Ordinary Diet (P/N: F9783B, Frontier Agricultural Science Newark, DE) into the base of 11.6 x 15.1 cm deli cups, lining them with a sheet of stiff plastic climbing mesh (P/N: VX620, Frost King by Thermwell, Mahwah, NJ), fitting them with aerated lids (P/N: D32CX, Anchor Packaging, Paragould, AR and P/N: FAB PPLID, Fabri-Kal, Kalamazoo, MI), and inverting them (so that the lid is now the base and the food sits at the ceiling). Rearing cups were populated with ~50 eggs and larvae fed *ad libitum* on Ordinary Diet from hatching to pre-pupation.

At the first sign of pre-pupation and wandering behavior (Wanderer stage), individuals were bathed with tap water and transferred to a 50 mL centrifuge tubes (P/N: C2750, MTC Bio, Metuchen, NJ) with a piece of a paper toweling (approximately 6 x 24 cm, P/N: 01804, Scott by Kimberly-Clark, Irving, TX) and topped with a modified cap that contained an 18% shade cloth insert to allow airflow. After 21 days, fully-formed pupae were sexed and added to same-sex tents for eclosion. Following eclosion, roughly ten male and ten female moths were placed into large tents, provided artificial nectar (P/N: 59144, The Gatorade Company, Chicago, IL), and allowed to mate freely. Resulting eggs were collected to renew the cycle.

The same conditions applied for experiments, with the exception that larvae were housed individually in Petri dishes (P/N: 351029, Corning, Corning, NY) from hatching through wanderer stage. Individual monitoring continues throughout pupation as usual, however instead of transferring pupae to group tents, fully formed pupa are transferred from their individual centrifuge tubes to an individual, eclosion cup that consisted of a deli cup with an aerated lid (P/N: D32CX, Anchor Packaging, Paragould, AR and P/N: FAB PPLID, Fabri-Kal, Kalamazoo,

MI) lined with a sheet of stiff plastic climbing mesh (P/N: VX620, Frost King by Thermwell, Mahwah, NJ). On the day of eclosion, individual, experimental moths were assigned a specific mate and each mate pair was transferred to a small mating tent containing artificial nectar (P/N: 59144, The Gatorade Company, Chicago, IL).

Horsenettle (*Solanum carolinense*)

Solanum carolinense (commonly Horsenettle, sometimes Devil's Tomato) is a member of the Solanaceae family and found in ephemeral habitats and agricultural fields throughout southeastern Canada and central and eastern United States (Britton and Brown 1913) (Fig. 1.3). It is a weedy, herbaceous perennial, that once established, spreads via horizontal rhizomes that can extend over a meter from the parent stem (Illnicki 1962), easing in the invasion and spread of newly colonized areas (Bassett and Munro 1986).

With the first frost of the autumn, horsenettle parts above the ground die marking the end of both the flowering and fruiting season. The below-ground parts overwinter and new shoots emerge early in the spring. Both growth and reproduction are indeterminate (Hardin et al. 1972). The flowers are approximately 3 cm in diameter, with five partially fused white to violet petals; five stamens with short filaments and large, fused yellow anthers (6–9 mm long) that surround the exerted pistil (Hardin et al. 1972). The flowers are visited by pollen-gathering bees, such as bumble bees, which must vibrate the flowers to remove pollen from the poricidal anthers (Hardin et al. 1972). Inflorescences consist of 1–20 flowers that mature acropetally. The fruit is a globose berry, smooth and glabrous, yellow or orange at maturity, 10–20 mm in diameter, and typically contain 60–100 seeds (Bassett and Munro 1986). The majority of the flowers are perfect and functionally hermaphroditic. However, some of the flowers, usually located at the tip of the

raceme, exhibit reduced non-functional pistils and are considered functionally staminate (Solomon 1985).

Most states list horsenettle as a noxious weed because of its ability to serve as a disease reservoir for important crops (e.g. tomato, potato, peppers, tobacco, eggplant) and because its extensive defense system is harmful to grazing livestock (e.g. horses, goats, cattle), therefore management of this weed can have significant economic consequences (Bassett and Munro 1986). The horsenettle defense system includes chemical defenses such as alkaloids, proteinase inhibitors, and phenolic compounds which guard the plant from both herbivores and pathogens (Campbell et al. 2013) and physical defenses, such as sharp spines which injure large herbivores (Bassett and Munro 1986). Specialist herbivores, such as tobacco hornworms (*Manduca sexta*) and the false potato beetle (*Leptinotarsa juncta*) have evolved to withstand the harsh chemistry (Wise 2007).

This study utilized progeny from rhizomes initially collected near State College, Pennsylvania (Mena-Ali et al. 2008). Sixteen original genets were collected by selecting plants a minimum of five meters apart. Resprouts of these cuttings were subjected to an 6-8 week vernalization period at 4˚C. Four rhizome segments were planted from each genet in 1-gallon pots within a greenhouse. Two of the resulting ramets from each genet received outcross-pollinations, while the remaining two received self-pollinations. Pollinations were performed using a custom, buzz-pollination device. Following fruit production, seeds were collected and germinated. Rhizomes from the resulting individuals have continuously been collected, vernalized, and resprouted.

Experimental plants were grown from rhizome cuttings planted in 1-gallon pots prepared with potting media (P/N: 1038500RG, Premier Tech Horticulture, Quakertown, PA) and fertilized with Osmacote Plus (P/N: 67-1525, ICL Specialty Fertilizers, Summerville, SC) and Micromax micronutrients (P/N: 67-1625, ICL Specialty Fertilizers, Summerville, SC). Plants

were maintained in a greenhouse under summer-like conditions (16:8 hour light:dark photoperiod, 26 ˚C, ambient humidity). Flowers were pruned to encourage verdant growth and Milstop (BioWorks, Victor, NY) was applied to discourage disease. All harvested leaves were triple rinsed with tap water to ensure any remaining Milstop was removed prior to experimentation.

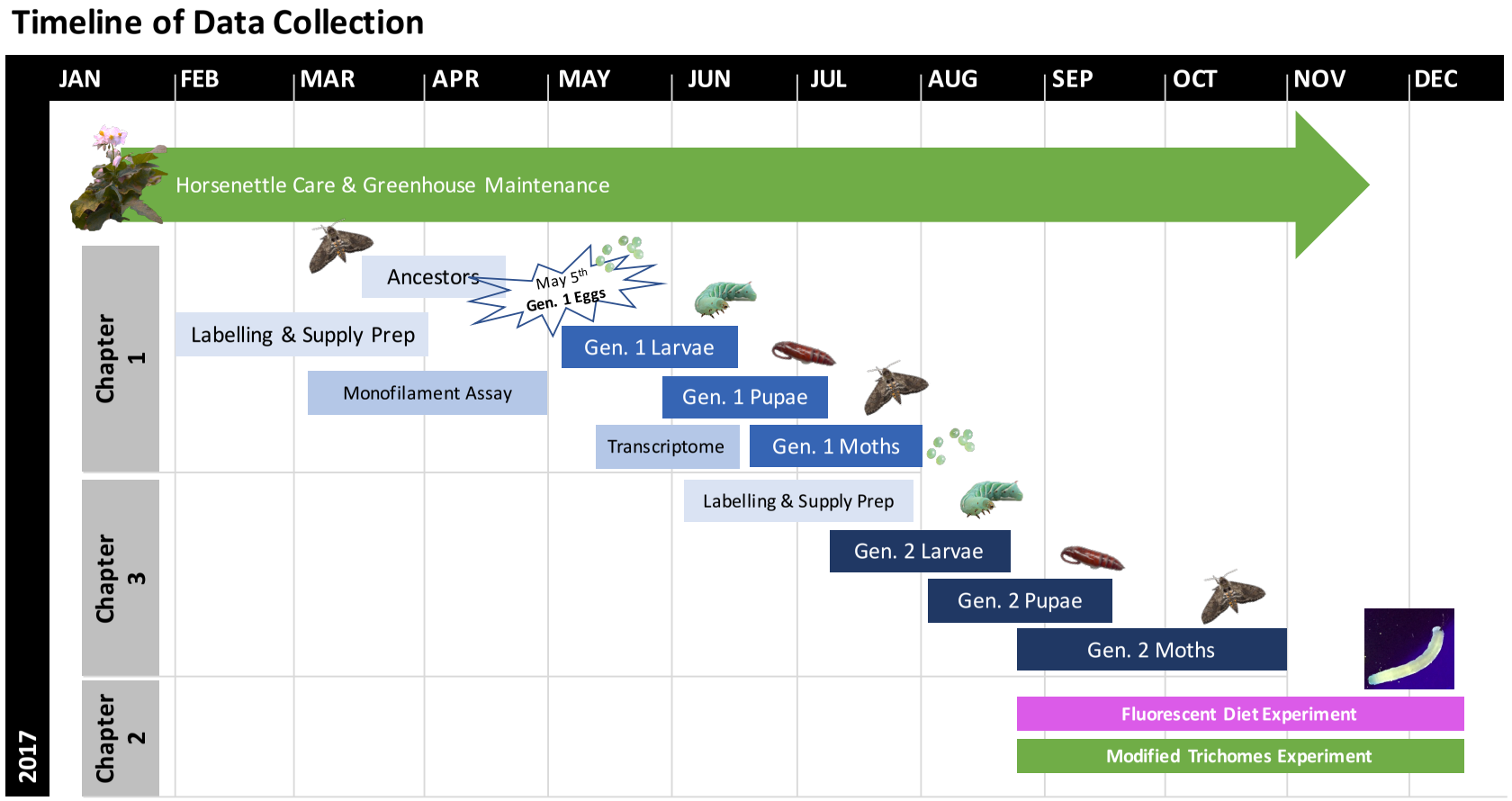

Figure 1-1: Timeline of Data collection.

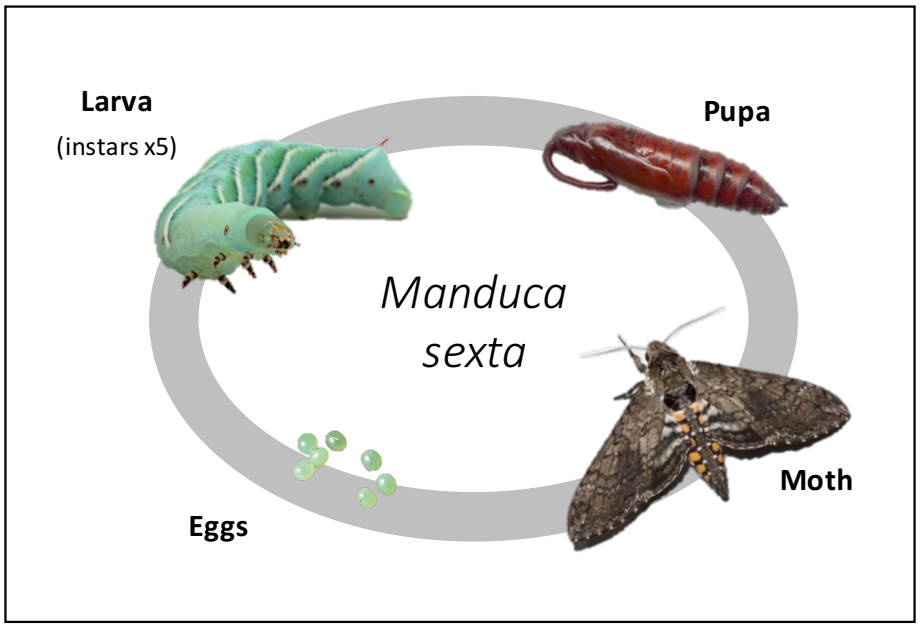

Figure 1-2: Tobacco Hornworm (*Manduca sexta*).

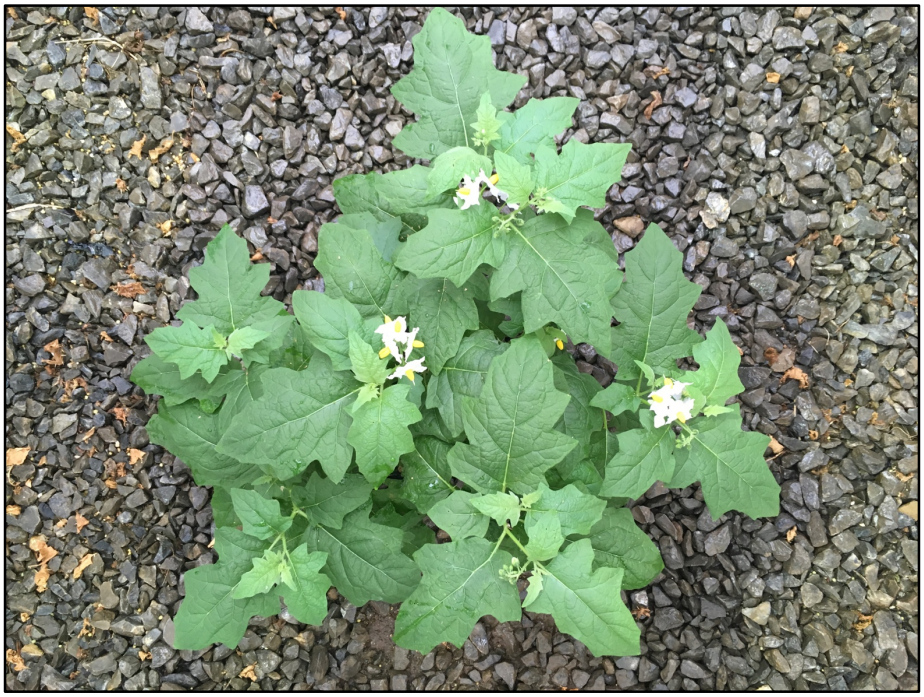

Figure 1-3: Horsenettle (*Solanum carolinense*).

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Chapter 2

Damage from non-glandular trichomes suppresses the growth and diet consumption of an herbivore

Abstract

While it is easy to study the effects of damage in plants, studies on the effects of damage in animals can be difficult to justify. However, herbivores routinely face damage by consuming plants that contain harmful mechanisms that protect the plant by deterring herbivory. These chemical and physical plant defenses are known to inhibit herbivore growth, survival, and reproduction. While the effects of consuming chemical defenses have been extensively studied, the long-term effects of consuming physical defenses are less understood due to the difficulties of separating them from the plant chemistry. Distinguishing the chemical and physical effects on herbivores improves the ability to predict outcomes of plant-insect interactions which can be useful when investigating plant breeding, adaptation, and evolution. Studies of one particular physical defense, non-glandular trichomes, often confound effects of trichomes with leaf chemistry and only study short-term effects. This study examined short and long-term effects of trichomes on tobacco hornworms (*Manduca sexta*) by using artificial diet and natural leaf treatments with the presence or absence of trichomes of horsenettle (*Solanum carolinense*). Larvae facing short-term exposure to treatments containing trichomes ate less diet, gained less mass, and produced less frass as compared to larvae exposed to treatments without trichomes. They also shifted more resources from growth to metabolic activity. The effect of trichomes was consistent across both diet types. Larvae facing long-term exposure to treatments containing trichomes also ate less diet, gained less mass, and produced less frass, but only when trichomes

were consumed on natural leaves and not when trichomes were consumed in artificial diet. In the long-term, trichomes reduced growth and increased development time, but they had no significant effect on survival or reproduction. Ultimately, trichomes should be considered a plant defense distinct from other physical and chemical defenses. Future work should expand upon the consequences of consuming trichomes and the long-term effects damage in animals.

Introduction

Damage is difficult to study. Apart from ethical justifications, it can be difficult to apply ecologically relevant levels of damage and measure their long-term effects. For these reasons, plants are often used to study the effects of damage on organisms. Herbivore damage imposes an important and almost constant pressure on plants. The consumption of leaf tissue by herbivores, including insects, reduces the amount of leaf area available for photosynthesis and inhibits the plant's ability to feed itself (Taiz and Zeiger 2010). Furthermore, the damage dealt by herbivores requires plants to divert their resources from growth to healing and defensive pathways (Taiz and Zeiger 2010, Fürstenberg-Hägg et al. 2013). As a result, plants that have experienced herbivory have fewer energetic resources available for growth, survival, and reproduction which limits their overall fitness (Taiz and Zeiger 2010, Kariyat et al. 2012, 2013). In order to inhibit these negative effects, plants have evolved extensive defense systems to counterattack their herbivorous assailants (Agrawal 1999, Taiz and Zeiger 2010, Mitchell et al. 2016). Therefore, a plant-based diet provides a natural opportunity to study the long-term effects of damage within an animal system.

The effects of plant defense systems can have far-reaching consequences throughout the life cycle of the herbivores by affecting growth, development time, survival, and reproduction (Maxell and Jennings 1980). In Lepidoptera, for example, larvae feed mainly on plant leaves and

stems (Gullan and Cranston 2011), which provide the mass and energy for immediate use by the growing larvae and also the necessary energy storage to complete metamorphosis and produce eggs (Gullan and Cranston 2011). Therefore, plant defenses consumed during lepidopteran juvenile phases have the potential to disrupt larval and subsequent life phases and reproduction.

Larvae that feed on diets containing plant defenses consume less leaf tissue and tend to be smaller than control larvae (Barbeta et al. 2008, Portman et al. 2015a), and also exhibit slower growth and delayed metamorphosis (Davidowitz et al. 2003). A longer development period extends the opportunity for larvae to be found by pathogens, predators, and parasitoids (Moran and Hamilton 1980, Krams et al. 2015). These factors can reduce the number of individuals surviving to adulthood, compounding reduced survivorship of larvae resulting from the plant defenses directly (Marston et al. 1975, Ojeda-Avila et al. 2003, Gog et al. 2014, Krams et al. 2015). Plant defenses, additionally, inhibit insect reproduction (Maxell and Jennings 1980), and these inhibitions are exacerbated through maternal effects including reduced nutritional stores within eggs (Roach and Wulff 1987) or inadequate energy to lay and distribute eggs (Raguso et al. 2007).

Long-lasting effects of plant defenses on herbivores can result from chemical and physical modes of defense. Chemical defenses directly reduce feeding through the unpalatability or toxicity of the leaves consumed by herbivores (Fraenkel 1959, Wittstock and Gershenzon 2002, Gog et al. 2014) and physical defenses limit herbivore feeding through traits such as leaf toughness (Turner 1994, Hanley et al. 2007), sharp spines (Milewski et al. 1991, Gowda 1996, Obeso 1997), and trichomes (Thurston 1970, Kennedy 2003). Of the physical plant defenses, trichomes appear unassuming and are easily overlooked by researchers. Trichomes are small hairs, typically only a few millimeters tall, extending from the epidermis of the plant. They are benign to large herbivores and are most commonly associated with defense against small insects (Levin 1973). Trichomes are highly diverse and classified as either glandular or non-glandular.

Glandular trichomes have a gland at the distal tip of the hair which can exude a sticky chemical to trap small herbivores like aphids or sequester toxins that poison larger herbivores (Levin 1973). Non-glandular trichomes do not have a gland but develop in a variety of architectures that are typically associated with physical rather than chemical modes of defense. For example, hook-shaped trichomes trap insects whereas straight trichomes impede insect movement or reduce oviposition events (Johnson 1953, Levin 1973, Pillemer and Tingey 1976, Eisner et al. 1998, Haddad and Hicks 2000, Fordyce and Agrawal 2001, Sletvold et al. 2010, Mitchell et al. 2016, Kariyat et al. 2017).

Some direct effects of consuming non-glandular trichomes have been documented. First instar larvae provided with outcrossed leaves with higher trichome densities were slower to initiate feeding as compared to larvae feeding on inbred leaves with lower trichome densities and lower concentrations of plant defense chemicals (Kariyat et al. 2012, 2017). Similarly, larvae were smaller and developed more slowly when fed on plants with non-glandular trichomes compared to other species or varieties with reduced trichome densities (Singh et al. 1971, Khan et al. 1986, Medeiros and Moreira 2002, Sletvold et al. 2010, Kariyat et al. 2017). However, in many of these studies, trichome density is confounded with leaf defensive chemistry, as there is an established correlation between these traits and are affected by species, variety, and inbreeding (Kariyat et al. 2013). For example, when trichome density increases in horsenettle (*Solanum carolinense*) following removal of leaf tissue by larvae, chemical defenses such as jasmonic acid precursors increase (Kariyat et al. 2013). This makes it somewhat challenging to disentangle the effect of trichomes and chemical defenses on herbivorous insects. There is some evidence that trichomes do have a specific effect. For example, horsenettle trichomes were excreted intact within the frass of first instar hornworms (*Manduca sexta*), and were found to have pierced the peritrophic membrane of dissected, third instar larvae (Kariyat et al. 2017). Associated damage to the midgut epithelium could trigger energetically costly response mechanisms as seen in effects

of leaf chemistry damage in the midgut (Pechan et al. 2002, Fescemyer et al. 2013). Allocation of resources to a damage response during the juvenile stage may have downstream consequences for metamorphosis and reproduction (Herms and Mattson 1992, Boggs 2009).

In this study, I perform a comprehensive examination of the long-term effects of consuming non-glandular trichomes across the entire life cycle of a Lepidoptera (*Manduca sexta*) from hatching to reproduction. To test for effects of trichomes in the absence of any effects of leaf chemistry, I included two artificial diet treatments, one with and one without added trichomes. To avoid confounding effects of leaf chemistry and trichome density, I added trichomes to an artificial diet. Furthermore, to examine the effects *in situ*, I used natural leaves of a single species of horsenettle (*Solanum carolinense*) and physically removed trichomes to establish natural leaf treatments with and without trichomes. I expected that, similar to the effects of chemical plant defenses, larvae that consume trichomes should consume less diet, have a lower body mass, take longer to mature to adulthood, be less likely to survive to adulthood, and produce fewer offspring. If these non-glandular, stellate trichomes are an effective component of the plant defense system, they should reduce herbivory on the plant and possibly have a net negative effect on the overall fitness of the herbivore. Overall, this system should provide a suitable opportunity to study long-term effects and trade-offs following natural damage in an animal.

Methods

Treatments

Larvae were obtained from my laboratory colony and subjected to an acute (48 hour) or chronic (from hatching to wandering) exposure to one of four treatment diets: 1) Ordinary Diet (artificial diet without trichomes added), Trichome Diet (artificial diet with trichomes added), 3) Shaved Leaves (natural leaves with most trichomes physically removed), and 4) Whole Leaves

(natural leaves with trichomes unmodified). My goal was to measure the effects of trichomes both *in situ* on natural leaves and in the absence of plant chemistry in artificial diet. The Ordinary Diet was a commercially-available artificial Tobacco Hornworm diet comprised of wheat-germ and agar and prepared according to the manufacturer's directions (P/N: F9783B, Frontier Agricultural Science, Newark, DE). The Trichome Diet was prepared by adding 1.5 mg of trichomes to each gram of Ordinary Diet; this ecologically relevant concentration was determined by identifying concentrations of trichomes that resulted in feeding rates that matched those on natural leaves (see Appendix). Trichomes were harvested by combining leaf tissue and palm-sized dry ice pieces within a cloth bag and shattering this tissue by shaking the bag. The resulting material was shifted through a fine mesh (90 μm) to separate the trichomes from leaf particles and dry ice. It should be noted that small particles of horsenettle may become airborne during this process, therefore it is highly recommended to wear appropriate personal protective equipment (PPE); including disposable gloves, face mask, and safety goggles. The Shaved Leaves were prepared by freshly harvesting natural leaves and removing 70-80% of the trichomes from the leaf cuticle on both the abaxial and adaxial surfaces using an electric razor (P/N: XA525/42, Norelco Axe by Philips, Amsterdam, Netherlands). The Whole Leaves were freshly harvested natural leaves. Treatment diets were freshly prepared at the start of each experiment. All animals were maintained on a 16:8 hour light:dark photoperiod at 26 ˚C with ambient humidity.

Acute Experiment

In order to examine the short-term effects of trichome consumption, I established an acute exposure experiment, in which larvae were reared on the same control diet until fifth instar and then exposed to treatment diets for 48 hours. Larvae for the acute experiment were reared from hatching in groups of ~50 larvae within rearing cups. Cups were assembled by pouring prepared Ordinary Diet (P/N: F9783B, Frontier Agricultural Science Newark, DE) into the base

of 11.6 x 15.1 cm deli cups, lining them with a sheet of stiff plastic climbing mesh (P/N: VX620, Frost King by Thermwell, Mahwah, NJ), fitting them with aerated lids (P/N: D32CX, Anchor Packaging, Paragould, AR and P/N: FAB PPLID, Fabri-Kal, Kalamazoo, MI), and inverting them (so that the lid is now the base and the food sits at the ceiling) Larvae fed *ad libitum* on Ordinary Diet from hatching to fifth instar. After molting to fifth instar, 110 larvae were transferred to individual, 100 x 10 mm Petri dishes (P/N: 351029, Corning, Corning, NY) and provided one of the four treatment diets *ad libitum* for 48 hrs. Initial larva mass and the mass of diet provided were measured to the nearest 0.001 g using an electronic pan balance. There was no significant difference in the initial mass of the acute experiment larvae assigned to the four different treatments ($F_{3,106} = 1.37$, $p = 0.257$).

After 48 hours of feeding on treatment diet, the mass of the larva, remaining diet, and all frass in the petri dish was measured to the nearest 0.001 g. These values allowed me to estimate larva growth during this period, the amount of diet they consumed, and the relative amount of frass they produced (amount of frass divided by the amount of diet consumed). I also calculated estimates of efficient conversion of diet), and metabolic gap. Conversion efficiency is the amount of ingested food converted to body weight (amount of larval weight gain divided by the amount of diet consumed). Metabolic gap is the proportion of diet consumed that is not accounted for by the amount of larval weight gain and the amount of frass produced (amount of diet consumed minus the amount of larval weight gain and the amount of frass produced; divided by the amount of diet consumed). Together, the metrics for frass, ECI, and metabolic gap account for the dispensation of all diet ingested by a larva.

Chronic Experiment

In order to examine the long-term effects of trichome consumption, I established a chronic exposure experiment in which larvae fed on treatment diets from hatching to the

wandering phase. Eggs were added to individual, 100 x 10 mm Petri dishes (P/N: 351029, Corning, Corning, NY); 617 hatched and were immediately provided one of the four dietary treatments. Natural leaf treatments were refreshed daily and artificial diet treatments were refreshed every three days. Larvae were monitored daily and the day of each molting event was recorded for every larva. Upon molting to fifth instar, larvae were transferred to individual, 150 x 20 mm Petri dishes (P/N: 82.1184.500, Sarstedt, Rommelsdorf, Germany). Fresh diet was provided and larvae and diet masses were recorded. After 48 hours of feeding on treatment diet, the mass of the larva, remaining diet, and all frass in the petri dish was measured to the nearest 0.001 g. I also calculated the same values as described for the larvae observed in the acute exposure experiment. Wet masses, as opposed to dry masses, were necessary to use because larvae could not be destructively sampled in order to monitor their growth through adulthood and reproduction. While it would have been possible to use dry masses in the Acute Exposure Experiment, wet masses were also used there in order to compare the effects of trichome exposure time. Larvae remained in the 150 x 20 mm Petri dishes and continued be provided their assigned treatment diets until they reached the wandering stage.

Individuals were bathed with tap water and transferred to a 50 mL centrifuge tubes (P/N: C2750, MTC Bio, Metuchen, NJ) with a piece of a paper toweling (approximately 6 x 24 cm, P/N: 01804, Scott by Kimberly-Clark, Irving, TX) and topped with a modified cap that contained an 18% shade cloth insert to allow airflow. After 21 days, pupae were transferred into individual, eclosion cups that consisted of a deli cup with an aerated lid (P/N: D32CX, Anchor Packaging, Paragould, AR and P/N: FAB PPLID, Fabri-Kal, Kalamazoo, MI) lined with a sheet of stiff plastic climbing mesh (P/N: VX620, Frost King by Thermwell, Mahwah, NJ). On the day of eclosion, individual moths were measured for mass, sexed, and assigned a mate from the same dietary treatment. Mating pairs were transferred to small mating tents containing artificial nectar (P/N: 59144, The Gatorade Company, Chicago, IL). Eggs were collected daily and photographed

alongside a ruler; total clutch size was counted for each mother and average egg volume was estimated by measuring the egg diameter (*V = ¾ * pi * r^3*) in ImageJ (Schneider, et al., 2012). Twenty-five eggs from each mother were weighed together and allowed to hatch in a 100 x 10 mm Petri dish to determine hatching success. Average egg weight was calculated by dividing the weight of each clutch by 25. All experiments were repeated in 2016 and 2017, but no annual effect was detected ($p > 0.05$).

Statistical Analyses

Statistical analysis of the effect of trichomes and natural base diet (nutrition and plant defense chemicals) on hornworms was performed in R (R Core Team 2014) using the '*lm*' function of the base package and the post hoc comparisons were performed using the '*HSD.tes*t' function from the agricolae package (de Mendiburu 2017). All models included trichome presence and base diet type as factors. The effects of trichomes, natural base diet (artificial vs natural), and exposure time (acute vs chronic) were evaluated using separate linear regression models for amount of diet consumed, larval mass gain, frass production, ECI, and metabolic gap. Each model included initial larval mass as a covariate to account for size-dependent effects, but omitting mass does not qualitatively change the results. It is expected that larger animals will consume more diet and produce more frass than smaller animals.

In order to assess the effects of trichomes and natural base diet on long-term growth, separate linear regression models were performed for mass at fourth instar, fifth instar, wandering, and eclosion. The effects of trichomes and natural base diet on development time and survival were evaluated using separate linear regression models for the time periods between early instars (hatching-to-fourth instar), late instars (hatching-to-wandering), pupation (wandering-to-eclosion), and complete development (hatching-to-eclosion). Larval mass was included as a covariate in the models for development time. The effects of trichomes and natural

base diet on reproduction were evaluated using separate linear regression models for the proportion of females (number of females that survived from the initial population), proportion of mothers (the number of females that laid at least one egg), clutch size, egg mass, egg volume, proportion of eggs that hatched. Female moth mass was included as a covariate in each model, except for the model evaluating the proportion of females.

Cohen's f^2 test for effect size was calculated for the effect of trichome presence, base diet and (where appropriate) exposure type. Cohen's recommended values were used to qualify effect size (Small effect: $f^2 > 0.02$, Moderate effect: $f^2 > 0.15$, Large effect: $f^2 > 0.35$) (Cohen 1988).

Results

Diet Consumption & Efficiency following Acute and Chronic Exposure

Diet consumption

The presence of trichomes in the diet significantly decreased the amount of diet that the larvae consumed during the experiment (12% decrease) (Trichome Presence (with or without trichomes): $t = -2.82$, $p < 0.001$; $f^2 = 0.132$; Fig. 2.1). This was true regardless of base diet type (Diet Type (artificial vs natural) x Trichome Presence: $t = 0.77$, $p = 0.441$; $f^2 = 0.689$; Fig. 2.1). Trichomes had a greater effect on diet consumption following an acute exposure (33% decrease) than following chronic exposure (6% decrease) to the treatment diet (Exposure Time (acute vs chronic) x Trichome Presence: $t = 3.811$, $p < 0.001$; $f^2 = 0.520$; Fig. 2.1). The type of base diet affected the amount of diet that larvae consumed, but only following chronic exposure to the treatment diet, when larvae feeding on natural-based diets consumed 88% more than larvae feeding on artificial-based diets; there was no effect of diet type following acute exposure (Exposure Time x Diet Type: $t = 6.087$, $p < 0.001$; $f^2 = 0.947$; Diet Type: $t = 0.063$, $p = 0.950$; $f^2 =$

0.689; Exposure Time: $t = 0.051$, $p = 0.959$; $f^2 = 0.322$; Fig. 2.1). There was no significant three-way interaction between Trichome Presence, Diet Type, and Exposure Time (Trichome Presence x Diet Type x Exposure Time: $t = -0.694$, $p = 0.488$; $f^2 = 0.947$; Fig. 2.1). Larger larvae consumed more diet than smaller larvae (Weight: $t = 23.01$, $p < 0.001$).

Larval mass gained

The presence of trichomes in the diet significantly limited the amount of mass that the larvae gained during the experiment (18% decrease) (Trichome Presence: $t = -6.31$, $p < 0.001$; $f^2 = 0.066$; Fig. 2.1). However, the effect of trichome presence on the amount of mass that the larvae gained was dependent upon both the diet type and the exposure time of the experiment (Trichome Presence x Diet Type x Exposure Time: $t = -2.73$, $p = 0.007$; $f^2 = 0.197$; Fig. 2.1). I therefore tested the two exposure types separately. Following acute exposure to the treatment diet, the presence of trichomes significantly decreased the amount of mass gained by the larvae (18% decrease), but this was more pronounced in the artificial diets (40% decrease), and larvae fed artificial-based diets gained more mass than did those fed natural-based diets (23% decrease) (Diet Type x Trichome Presence: $t = 3.65$, $p < 0.001$; $f^2 = 0.943$; Trichome Presence: $t = -8.70$, $p < 0.001$; $f^2 = 0.755$; Diet Type: $t = -5.76$, $p < 0.001$; $f^2 = 0.319$) Conversely, following chronic exposure the presence of trichomes in either diet type did not significantly affect mass gain, but the natural-based diets led to larvae gaining 6% more mass (Trichome Presence: $t = 0.27$, $p = 0.788$; $f^2 = 0.036$; Diet Type: $t = 2.75$, $p = 0.006$; $f^2 = 0.036$; Diet Type x Trichome Presence: $t = -0.48$, $p = 0.629$; $f^2 = 0.037$). Larger larvae gained more mass than smaller larvae (Weight: $t = 18.57$, $p < 0.001$).

Diet excreted as frass

The presence of trichomes in the diet led to a 13% decrease in the proportion of frass that the larvae produced during the experiment (Trichome Presence: $t = -3.76$, $p < 0.001$; $f^2 = 0.069$; Fig. 2.1). This was true regardless of base diet type (Diet Type x Trichome Presence: $t = 0.46$, $p = 0.649$; $f^2 = 0.690$), but depended upon the type of exposure (Exposure Time x Trichome Presence: $t = 2.80$, $p = 0.005$; $f^2 = 0.070$; Fig. 1.1); the presence of trichomes had a weaker effect on frass production for larvae with chronic exposure (3% decrease) to the treatment diets than following an acute exposure (22% decrease). Larvae feed on natural-based diets produced 63% more frass than larvae feed on artificial-based diets, regardless of exposure time (Diet Type: $t = 6.96$, $p < 0.0001$; $f^2 = 0.672$; Exposure Time x Diet Type: $t = -0.27$, $p = 0.786$; $f^2 = 0.683$; Fig. 2.1). Exposure Time alone did not affect how much frass larvae produced, and there was no three-way interaction between Trichome presence, Diet type, and Exposure time (Trichome Presence x Diet Type x Exposure Time: $t = -1.37$, $p = 0.172$; $f^2 = 0.701$; Exposure Time: $t = -1.25$, $p = 0.499$; $f^2 = 0.018$; Fig. 2.1). Larger larvae produce more frass than smaller larvae (Weight: $t = 16.03$, $p < 0.001$).

Conversion efficiency

The presence of trichomes in the diet led to a 6% decrease in the efficiency with which larvae converted diet to body mass following the experiment (Trichome Presence: $t = -2.47$, $p = 0.014$; $f^2 = 0.054$; Fig. 2.1). However, the effect of trichome presence on conversion efficiency was dependent upon both the diet type and the exposure time of the experiment (Trichome Presence x Diet Type x Exposure Time: $t = -2.57$, $p = 0.010$; $f^2 = 1.265$; Fig. 2.1). I therefore tested the two exposure types separately. Following acute exposure, trichomes tended to reduce conversion efficiency, but this did not achieve statistical significance, and natural-based diets decreased conversion efficiency by 25% (Trichome Presence: $t = -1.82$, $p = 0.072$; $f^2 = 0.033$; Diet Type: $t = -4.46$, $p < 0.001$; $f^2 = 0.247$; Diet Type x Trichome Presence: $t = 1.41$, $p = 0.160$; f^2

= 0.260). Following chronic exposure, trichomes did not significantly affect conversion efficiency, and natural-based diets decreased conversion efficiency by 43% (Trichome Presence: $t = 0.47$, $p = 0.638$; $f^2 = 0.006$; Diet Type: $t = -20.51$, $p < 0.001$; $f^2 = 1.755$; Diet Type x Trichome Presence: $t = -1.55$, $p = 0.122$; $f^2 = 1.789$). Larger larvae had lower conversion efficiency than smaller larvae (Weight: $t = -3.45$, $p < 0.001$).

Metabolic gap

The presence of trichomes in the diet significantly increased the metabolic gap (41% increase) of the larvae gained following the experiment (Trichome Presence: $t = 4.21$, $p < 0.001$; $f^2 = 0.086$; Fig. 2.1). The effect of trichome presence on the metabolic gap was dependent upon both the natural diet and the exposure time of the experiment (Trichome Presence x Diet Type x Exposure Time: $t = 2.452$, $p = 0.014$; $f^2 = 0.087$; Fig. 2.1). I therefore tested the two exposure types are separately. Following acute exposure, trichomes increased the metabolic gap by 71%, regardless of base diet type, and natural-based diets did not significantly affect the metabolic gap (Trichome Presence: $t = 3.33$, $p = 0.001$; $f^2 = 0.120$; Diet Type: $t = -1.18$, $p = 0.243$; $f^2 = 0.077$; Diet Type x Trichome Presence: $t = -1.00$, $p = 0.322$; $f^2 = 0.188$). Following chronic exposure, trichomes increased the metabolic gap, only when left *in situ* on natural leaves (59% increase), not when incorporated into artificial diet (Diet Type x Trichome Presence: $t = 2.63$, $p = 0.009$; $f^2 = 0.052$; Trichome Presence: $t = 0.94$, $p = 0.347$; $f^2 = 0.039$). Larger larvae had smaller metabolic gaps than smaller larvae (Weight: $t = -10.21$, $p < 0.001$).

Size, Development Time, & Survival following Chronic Exposure

Size at Selected Phenophases

The presence of trichomes in the diet significantly decreased the mass of fourth instar larvae, but only when fed on the natural-based diets (19% decrease) (Diet Type x Trichome Presence: $t = -4.53$, $p < 0.001$; $f^2 = 0.106$; Trichome Presence: $t = 1.17$, $p = 0.244$; $f^2 = 0.050$; Fig. 2.2). Larvae that fed on the natural-based diet were 20% larger at fourth instar than larvae that fed on artificial-based diets (Diet Type: $t = 7.54$, $p < 0.001$; $f^2 = 0.102$; Fig. 2.2).

The same trends were observed when larvae reached fifth instar. Trichomes restricted larvae mass when fed on natural-based diets only (12% decrease) (Diet Type x Trichome Presence: $t = -4.51$, $p < 0.001$; $f^2 = 0.195$; Trichome Presence: $t = 2.52$, $p = 0.012$; $f^2 = 0.038$; Fig. 2.2) and larvae that fed on the natural-based diets had 29% greater mass at fifth instar than larvae that fed on artificial-based diets (Diet Type: $t = 9.90$, $p < 0.001$; $f^2 = 0.198$; Fig. 2.2).

When larvae reached wandering, the presence of trichomes in the diet significantly decreased larval mass only when consumed as part of the natural-based diet (6% decrease) (Diet Type x Trichome Presence: $t = -3.38$, $p < 0.001$; $f^2 = 0.057$; Trichome Presence: $t = 1.57$, $p = 0.117$; $f^2 = 0.024$). Diet type did not affect larval mass at wandering (Diet Type: $t = -0.22$, $p = 0.825$; $f^2 = 0.049$; Fig. 2.2)

After pupation, moths that had been reared on natural diet containing trichomes had 13% lower mass than those reared on trichome-free natural diet, whereas the mass of moths was not affected by consuming trichomes in an artificial diet (Diet Type x Trichome Presence: $t = -3.05$, $p = 0.002$; $f^2 = 0.272$; Trichome Presence: $t = 1.15$, $p = 0.252$; $f^2 = 0.024$; Fig. 2.2). Overall, moths that had been reared on natural-based diets had 23% lower mass than those reared on artificial-based diets (Diet Type: $t = -4.22$, $p < 0.001$; $f^2 = 0.204$; Fig. 2.2).

Development Time

The development time of early instar larvae (number of days between hatching and molting to fourth instar) was not significantly affected by trichome presence in the diet, diet type,

or larva weight (Trichome Presence: $t = 0.09$, $p = 0.926$; $f^2 = 0.007$; Diet Type: $t = 0.87$, $p = 0.383$; $f^2 = 0.015$; Diet Type x Trichome Presence: $t = 1.36$, $p = 0.176$; $f^2 = 0.021$; Weight: $t = -0.31$, $p = 0.759$; Fig. 2.2). However, in later instars both diet type and weight significantly affected larval development time from hatching to wandering (Diet Type: $t = 2.02$, $p = 0.044$; $f^2 = 0.028$; Weight: $t = -8.57$, $p < 0.001$); larvae that fed on natural-based diets required 0.41 more days to reach wandering and larger larvae required fewer days to reach wandering (Fig. 2.2). The presence of trichomes in the diet did not significantly affect larval development time, regardless of diet type (Trichome Presence: $t = 0.17$, $p = 0.866$; $f^2 = 0.003$; Diet Type x Trichome Presence: $t = 0.87$, $p = 0.385$; $f^2 = 0.032$; Fig. 2.2). Larger larvae required fewer days to reach wandering than smaller larvae (Weight: $t = -8.57$, $p < 0.001$).

Trichomes in the diet significantly increased the number of days required for pupation, but only when larvae were reared on artificial-based diets (1.78 days or 6%) (Diet Type x Trichome Presence: $t = -2.00$, $p = 0.046$; $f^2 = 0.025$; Trichome Presence: $t = 3.33$, $p < 0.001$; $f^2 = 0.027$; Fig. 2.2). Diet Type alone did not significantly affect the number of days required for pupation (Diet Type: $t = 1.88$, $p = 0.060$; $f^2 = 0.011$). Larger larvae required more days to pupate than smaller larvae (Weight: $t = 4.25$, $p < 0.001$).

Overall development from hatching to adulthood was significantly delayed by the presence of trichomes in the diet (0.93 days or 2%) (Trichome Presence: $t = 2.90$, $p = 0.004$; $f^2 = 0.021$; Fig. 2.2), regardless of base diet type (Diet Type x Trichome Presence: $t = -1.45$, $p = 0.148$; $f^2 = 0.027$). Diet type did not affect the total number of days from hatching to adulthood (Diet Type: $t = 1.84$, $p = 0.067$; $f^2 = 0.008$). Larger moths required more days to develop than smaller moths (Weight: $t = 3.50$, $p < 0.001$).

Survival

Survival of early instar larvae (number of larvae surviving from hatching to fourth instar) was not significantly affected by trichome presence or diet type (Trichome Presence: t = -0.56, p = 0.575; f^2 = 0.008; Diet Type: t = -1.09, p = 0.278; f^2 = 0.014; Diet Type x Trichome Presence: t = -1.12, p = 0.262; f^2 = 0.022; Fig. 2.2). Trichome presence had no significant effect on overall larval survival (number of larvae surviving from hatching to wandering), but natural-based diets decreased larval survival by 14% (Trichome Presence: t = -0.71, p = 0.481; f^2 = 0.005; Diet Type: t = -2.99, p = 0.003; f^2 = 0.039; Diet Type x Trichome Presence: t = -0.61, p = 0.544; f^2 = 0.047; Fig. 2.2).

During pupation, pupae survival was not significantly affected by trichome presence (Trichome Presence: t = -0.62, p = 0.534; f^2 = 0.006; Diet Type x Trichome Presence: t = 1.60, p = 0.110; f^2 = 0.034; Fig. 2.2). While not significant, pupae that had fed on natural-based diets tended to show 13% increased survival over those raised on artificial diets (Diet Type: t = 1.51, p = 0.133; f^2 = 0.032; Fig. 2.2). Survival from hatching to adulthood of all individuals (male and female) was not affected by trichome presence or diet type (Trichome Presence: t = -0.91, p = 0.366; f^2 = 0.001; Diet Type: t = -1.05, p = 0.293; f^2 = 0.002; Diet Type x Trichome Presence: t = 0.730, p = 0.463; f^2 = 0.003; Fig. 2.2).

Reproduction following Chronic Exposure

The presence of trichomes in the diet significantly decreased the number of females that survived to adulthood by 10% (Trichome Presence: t = -2.03, p = 0.043; f^2 = 0.007; Fig. 2.3), regardless of base diet type (Diet Type x Trichome Presence: t = 1.66, p = 0.098; f^2 = 0.070). The number of females surviving to adulthood was not affected by base diet type (Diet Type: t = -0.41, p = 0.681; f^2 = 0.010).

The number of females that produced eggs was not significantly affected by the presence of trichomes in the diet or by the base diet type, however there was a 6% decrease in the number of females reared on trichome-containing diets that laid any eggs (Trichome Presence: $t = -0.04$, $p = 0.967$; $f^2 = 0.001$; Diet Type: $t = -1.05$, $p = 0.293$; $f^2 = 0.011$; Diet Type x Trichome Presence: $t = -0.27$, $p = 0.785$; $f^2 = 0.013$; Fig. 2.3). Moth weight had no significant effect on whether or not a female laid any eggs (Weight: $t = 1.63$, $p = 0.105$).

The total number of eggs laid by a female was not significantly affected by the presence of trichomes in the diet or by the base diet type, however there was a 12% increase in the number of eggs produced by mothers reared on trichome-containing diets (Trichome Presence: $t = 0.58$, $p = 0.564$; $f^2 = 0.004$; Diet Type: $t = 1.35$, $p = 0.180$; $f^2 = 0.017$; Diet Type x Trichome Presence: $t = 0.10$, $p = 0.924$; $f^2 = 0.025$; Fig. 2.3). Larger mothers laid more eggs (Weight: $t = 3.11$, $p = 0.002$).

The average weight of a single egg was not significantly affected by the presence of trichomes in the diet, the base diet type, or moth weight (Trichome Presence: $t = 1.09$, $p = 0.279$; $f^2 = 0.010$; Diet Type: $t = -1.46$, $p = 0.146$; $f^2 = 0.022$; Diet Type x Trichome Presence: $t = -0.14$, $p = 0.891$; $f^2 = 0.028$; Weight: $t = 1.64$, $p = 0.103$; Fig. 2.3).

The volume of a single egg was not affected by the presence of trichomes in the diet, regardless of base diet type (Trichome Presence: $t = 0.42$, $p = 0.675$; $f^2 = 0.002$; Diet Type x Trichome Presence: $t = -0.05$, $p = 0.964$; $f^2 = 0.070$). Single-egg volume was significantly affected by the base diet type consumed by the mother (Diet Type: $t = -2.72$, $p = 0.007$; $f^2 = 0.070$; Fig. 2.3); mothers that had fed on natural-based diets laid 7% smaller eggs by volume (Fig. 2.3). Weight of mothers did not significantly affect egg volume (Weight: $t = -1.05$, $p = 0.296$; Fig. 2.3).

The proportion of eggs that hatched was not significantly affected by the presence of trichomes in the diet, the base diet type, or moth weight (Trichome Presence: $t = 1.55$, $p = 0.123$; $f^2 = 0.013$; Diet Type: $t = 0.32$, $p = 0.749$; $f^2 = 0.008$; Diet Type x Trichome Presence: $t = -1.13$, p

= 0.259; f^2 = 0.014; Weight: t = 0.67, p = 0.507; Fig. 2.3). However, 5% more eggs hatched from mothers reared on trichome-containing diets.

Discussion

Horsenettle trichomes had immediate and long-term effects on hornworms. This largely remained true whether trichomes remained *in situ* on natural leaves or were added to artificial diets. With a pair of natural leaf treatments with and without trichomes *in situ* and a pair of artificial diet treatments with and without added trichomes, the effects of non-glandular, stellate trichomes on hornworm feeding, growth, development, and reproduction were examined. Additionally, comparisons between the natural and artificial base diet types hint at the effects of differences in leaf chemistry and nutrition. The presence of dietary trichomes reduced larval feeding, weight gain, and relative frass production following acute (48 hour) exposure of naïve fifth instar larvae to treatment diets and, to a lesser extent, following chronic exposure to treatment diets from hatching. Furthermore, trichomes consumed by larvae led to a decrease in conversion efficiency and an increase in metabolic gap. Trichomes only affected conversion efficiency when attached to natural leaves, not when consumed within artificial diet and trichomes affected conversion efficiency to a larger extent following chronic exposure as compared to following acute exposure. After acute exposure to treatment diets, trichomes increased the metabolic gap, regardless of diet type whereas after chronic exposure, only trichomes on natural leaves increased the metabolic gap.

Over time, the effects of trichomes on consumption and metabolic efficiency have downstream effects on larval size, development time, and survival. Rather than investing resources in growth, larvae facing injury from trichomes divert resources to metabolically active processes, including tissue repair. As a result, larvae reared on natural leaves with trichomes were

smaller than larvae reared on natural leaves without trichomes at fourth instar, fifth instar, wandering, and eclosion. This is important because the mass of an individual can influence their development time, and smaller larvae tend to require more time to reach metamorphosis (Davidowitz et al. 2003). In this study, greater larvae mass was associated with increased consumption, weight gain, frass production, and metabolic gap, but a lower ECI. Larger larvae reached the wandering phase more quickly than smaller larvae, but were slower to complete metamorphosis, leading to an overall longer time to reach maturity.

Trichomes present in the diet did not affect overall larval development time from hatching to adulthood, however pupae that had been reared on artificial diet with trichomes required a longer metamorphosis phase which extended overall development to maturity by two days. For offspring of parents not reared on trichomes, this additional time could provide a head start in the competition for resources in the next generation, either by providing more time to establish themselves on a plant host when competition density is lower or by exposing later offspring (from parents reared on trichome-containing diets) to more extreme plant defenses induced by the feeding of earlier offspring (McClure 1980, Livdahl 1982, Agrawal 1999). Surprisingly, trichomes present in the diet did not affect overall survival to adulthood. It is important to note that survival may have been enhanced in this experiment due to the laboratory conditions which shielded larvae from exposure to predators, pathogens, or parasitoids, as would be expected for wild populations (Moran and Hamilton 1980, Krams et al. 2015). The lengthened development time for larvae reared on diets containing trichomes would potentially increase the exposure of larvae to these environmental pressures and likely increase the difference in survival between treatments.

In contrast to their effects on overall survival, trichomes present in the diet affected the proportion of females surviving to adulthood, with fewer females surviving to adulthood in populations reared on trichome-containing treatments, regardless of diet type. Trichomes present

in the diet did not significantly affect any other reproduction traits, however, there was a trend for trichomes in both artificial-based and natural-based diets to lead to larger clutches with larger, heavier eggs. Moths reared on natural leaves with trichomes were smaller than moths reared on natural leaves without trichomes and moth weight affected the number of eggs laid by each mother, with larger mothers laying more eggs than smaller mothers. Finally, although not quantified, it was noted that the color of the eggs varied between treatments. Mothers reared on artificial-based diets clearly laid blue eggs with slight variation between treatments with and without trichomes, while mothers reared on natural base diets laid bright green eggs, again, with slight variation between treatments with and without trichomes. Together, differences in egg color, size, and number hint at the possibility of variation in egg provisioning that could affect the next generation. Heavier eggs are associated with faster development and larger offspring, so this potential size discrepancy could indicate that trichomes are enough of an environmental pressure to trigger maternal effects that prepare the next generation to face similar conditions (Rossiter 1991). While none of the individual variables were greatly affected by treatment diet, when considering them together, they could have serious implications at the population level. Extrapolations of the number of mothers, clutch sizes, and hatch rates hypothesizes that both trichomes and natural leaves reduce the number of offspring starting the next generation. Trichomes reduced the number of offspring of by 5% when consumed in artificial diet and by 7% when consumed on natural leaves. Natural leaf consumption reduced the number of offspring produced by 9%. For now, this study only hints at the effects of trichomes on reproduction and future work will be needed to fully examine the population level effects as well as the maternal effects of trichomes.

Exposure time alone (48 hours versus throughout development) did not affect larval diet consumption and metabolic efficiency, but it may have provided insights into the mechanisms of trichomes. Larvae given an acute exposure were naïve to plant defenses prior to trichome

exposure at fifth instar. In the chronic experiment, however, larvae were exposed to trichomes from hatching to fifth instar and it is possible that these larvae had the opportunity to upregulate the appropriate damage response genes prior to the consumption and efficiency measurements at fifth instar (Jarosz 1993, Alberts et al. 2004). Alternatively, it is possible that early exposure to trichome-containing diets culled weaker larvae at earlier instars in the chronic experiment, so that by the measurements at fifth instar only the healthiest larvae had survived (Darwin 1859). However, there was no significant difference in early larval survival between larvae exposed and not exposed to trichomes during the chronic exposure experiment. Therefore, it is more likely that the magnitude of differences between acute and chronic trichome exposure are due to an adaptive response such as differences in gene regulation and not due to differences in survival.

Overall, trichomes have a consistent, negative effect on larval consumption and efficiency regardless of exposure type. This may be due to the physical nature of trichomes. Unlike most other leaf tissues, trichomes are not digestible and are excreted intact within the frass (Kariyat et al. 2017). Therefore, trichomes are assumed to be nutritionally unavailable to larvae. Trichomes present in the diet may act as voids that essentially reduce the nutritional density of diets, forcing larvae to expend more time and energy feeding (Davidowitz et al. 2003, Ojeda-Avila et al. 2003, Portman et al. 2015b). If this were the case, larvae reared on trichome-containing diets may be smaller or show less efficiency in converting diet to body mass and larvae may compensate for the reduced nutritional density by consuming more diet. However, larvae reared on trichome-containing diets actually consumed less diet, indicating that trichomes made it more difficult for larvae to consume or process diet. Furthermore, larvae reared on trichome-containing diets excreted a smaller proportion of the diet as frass and actually converted a lower proportion of the retained diet to body mass.

The needle-like shape of trichomes, which is retained even after trichomes pass intact through the larvae (Kariyat et al. 2017), makes it highly-likely that trichomes at least scrape and

catch on the walls of the larval gut. One indication of this is the lower proportion of frass production. Trichomes catching on the larval gut lining could impede the progress of the food bolus, which might allow more time for water and nutrient absorption and decrease the frass mass. While not quantified, frass pellets from trichome-fed larvae was observed to be smaller and more desiccated than frass pellets from larvae not exposed to trichomes. If trichomes impede the physical progress of food through the larvae, it is unlikely that larvae would be able to accelerate the gut bolus even after chronic exposure and would account for the consistency between exposure times.

One other factor to consider is the fact that instead of being excreted or converted, the diet was likely consumed in other metabolic processes as noted by the increased metabolic gap. This pattern of low conversion efficiency and high metabolic gap is less indicative of poor nutrition and more indicative of illness or injury (Freitak et al. 2003, Rauw 2012). Physical damage from trichomes is an alluring theory based on the above data. The larval midgut may be particularly susceptible to damage because, unlike the foregut or hindgut, it is not protected by a waxy cuticle layer (Gullan and Cranston 2011). Horsenettle trichomes have been shown to catch on the peritrophic membrane which surrounds the food bolus as it moves along the alimentary canal (Kariyat et al. 2017). This layer acts as a sieve to restrain large particles while allowing nutrients in the diet to flow through to the midgut epithelium (Gullan and Cranston 2011). Damage to the peritrophic membrane is of little consequence, because it is not the barrier separating the digestive tract from the open circulatory system and because it is regularly shed in the frass as the food bolus moves through the larvae, including when larvae molt between instars (Gullan and Cranston 2011). However, if trichomes can press through the peritrophic membrane, they may pierce the surrounding epithelium. This layer is the site of nutrient absorption and separates the gut lumen from the hemocoel (Gullan and Cranston 2011). Damage to this tissue would allow food particles, and any associated microbes, to leak through into the hemolymph

(Gullan and Cranston 2011). In this scenario, reduction in growth and conversion efficiency may be explained by a metabolic upregulation of immune response pathways to defend against contamination and infections (Freitak et al. 2003, Rauw 2012). Physical damage of midgut tissues would also need to be repaired as indicated by studies of chemical damage to the midgut epithelium of fall armyworm larvae following consumption of corn leaves, which revealed an upregulation of genes associated with tissue repair and digestive enzymes (Pechan et al. 2002, Fescemyer et al. 2013).

Even after a longer exposure to trichomes, larvae would not have been able to shield themselves from potential physical damage. While the foregut and hindgut are protected by a chitin layer, the midgut cannot be shielded in the same manner without reducing nutrient absorption across the epithelium (Gullan and Cranston 2011). However, following chronic exposure, larvae could respond by upregulating the appropriate suites of genes to manage the damage. This may explain the discrepancy in magnitude for trichome effect in which trichomes had a greater effect after acute exposure than after chronic exposure. Future work will be necessary to understand exactly which metabolically taxing processes were upregulated following the consumption of both trichomes and natural-based diet. For now, this study can only connect the overall increase in metabolic gap with its knock-on effects on larval size, development time, survival, and reproduction.

The type of base diet (natural or artificial) affected diet consumption only following chronic exposure, with larvae reared on the natural-based diet consuming nearly twice the amount of diet as larvae reared on artificial-based diets. Larvae exposed to natural-based diets gained less mass than larvae exposed to artificial-based diets after acute exposure, but larvae reared on natural-based diets gained more mass than larvae reared on artificial-based diets after chronic exposure. In the case of frass production, larvae exposed to natural-based diets produced relatively more frass than larvae exposed to artificial-based diets, regardless of exposure length.

Larvae exposed to natural-based diets had significantly lower conversion efficiency than larvae exposed to artificial based diets, but diet type had no effect on metabolic gap.

Following chronic exposure, larvae reared on natural-based diets were larger than larvae reared on artificial-based diet in the larval phase, however following metamorphosis, moths reared on natural-based diets were smaller than moths reared on artificial-based diet. Natural-based diets extended the larval development phase, but not the pupation phase. So, overall, natural-based diets only extended development from hatching to maturity by one day. Natural based diets decreased larval survival to adulthood, but had no effect on the proportion of females surviving to adulthood. Diet type did not significantly affect the proportion of females laying eggs, the number of eggs laid by each female, or the weight of each egg; however, mothers reared on natural-based diets laid significantly smaller eggs than mothers reared on artificial-based diets. As suggested above, this may indicate that the base diet type may have the potential to induce maternal effects in the next generation.

Unlike the consistent, negative effect of trichomes, the effect of base diet type varied between exposure times. After acute exposure, larvae provided with natural-based diets consumed less diet and gained less mass than larvae provided with artificial-based diets. However, after chronic exposure, larvae provided with natural-based diets actually consumed more diet and gained an equal amount of mass as compared to larvae provided with artificial-based diets. conversion efficiency and metabolic gap were lower for larvae exposed to natural-based diets as compared to larvae exposed to artificial diets. More importantly, larvae chronically exposed to natural-based diets exhibited lower conversion efficiency and higher metabolic gaps as compared to acutely exposed larvae. As in the case of the effect of trichome consumption, this could be the result of differences in survival. It is clear from this experiment that larvae reared on natural-based diets are less likely to survive than larvae reared on artificial-based diets. It could be that the smaller, weaker larvae were culled at earlier instars, leaving the healthier, more

efficient larvae to survive and represent the natural-based diet groups in the consumption and efficiency measurements at fifth instar. Larvae reared on natural-based diet were larger than peers reared on artificial-based diets and there is some indication of this during the chronic experiment in which larger larvae ($t = 5.71$, $p < 0.001$) and larvae with smaller metabolic gaps ($t = -4.39$, $p < 0.001$) were more likely to survive between fourth and fifth instar. However, without mass and metabolic efficiencies for earlier instars it is difficult to measure the ability of early survival to affect the representative population of later instars. Alternatively, as in the case of trichomes, chronic exposure to natural-based diets may provide larvae with the opportunity to upregulate suites of genes that more efficiently process diet (Jarosz 1993, Alberts et al. 2004). Rather than a physical damage response, natural-based diets may require the upregulation of metabolic pathways capable of metabolizing some of the alkaloids and other toxins within leaves (Wink and Theile 2002). This would explain the lower conversion efficiency and higher metabolic gaps of chronically exposed larvae as compared to acutely exposed larvae.

It should be noted that the difference between natural-based and artificial-based diets is not limited to defensive chemistry. Artificial diets, which tend to be oligidic (chemically undefined), never mimic the complexity of natural leaves and the natural-based diet in these experiments likely contain a more complete roster of nutrients than the artificial base diet (Cohen 2001). The relatively larger size of larvae reared on natural-based diets as compared to larvae reared on artificial-based diets may be due a nutritional boost the chronically-exposed larvae gained by feeding on leaves from hatching (Davidowitz et al. 2003, Ojeda-Avila et al. 2003, Barbeta et al. 2008, Gog et al. 2014, Portman et al. 2015a). Larvae only acutely-exposed to natural-based diets may not have had time to benefit from enhanced nutrition, but faced the immediate effects of the defensive chemistry, including reduced weight gain (Barbeta et al. 2008, Portman et al. 2015a).

Indeed, the combination of rich nutrition of natural leaves and lack of trichomes in the Shaved Leaf treatment seems to have benefited larvae in this experiment. Larvae reared on Shaved Leaves gained the most weight, had the lowest metabolic gap, and developed the fastest. In some cases, developmental lags due to insufficient growth may cause larvae to molt to a sixth instar which allows larvae to gain more weight prior to metamorphosis (Kingsolver 2007). In this study, there was a slight trend in which every treatment included a least four larvae that required an extra instar, with the exception of the Shaved Leaf treatment, which had no extra instar larvae (X^2 (3, n = 310) = 3.03, p = 0.38). It is possible that the Shaved Leaves provided adequate nutrition without the hindering effects of trichomes.

The base diet type also affected the efficacy of trichomes. Trichomes *in situ* on natural leaves were more effective when reducing diet consumption, larval weight gain, and conversion efficiency (while increasing metabolic gap), as compared to trichomes removed from the leaf epidermis and incorporated into artificial diet. This was particularly evident after chronic exposure. It may be that the orientation of trichomes, relative to the chewing larvae determines the efficacy of trichomes. On natural leaves, trichomes remain perpendicular to the food surface and the larval mouth (Levin 1973). In the artificial diet, trichomes, previously removed from leaves and then mixed into artificial diet, were randomly oriented within the interior of the food and may have been broken or consumed parallel to the larval mouth. Alternatively, the efficacy of trichomes may be determined by the combination of trichomes and leaf defensive chemistry. If trichomes pierce the larval midgut, the defensive chemicals within the natural leaves could leak outside of the gut, allowing chemical damage to vulnerable cavities within the larval bodies (Pechan et al. 2002, Fescemyer et al. 2013). This could lead to more damaging effects than if piercing trichomes allowed artificial diet, which lacks defensive chemicals, to leak into the same area.

In some cases, like survival, trichomes showed a trend that did not cross the threshold of significance. This may be due, in part, to the method for removing trichomes during the preparation of the Shaved Leaves treatment. The method used in this study was not fully effective, and a small proportion of trichomes remained on the leaf surfaces. As such, with a more complete removal method, the effect of trichomes may be more confidently measured with a clearer distinction between treatments. Furthermore, survival may have been enhanced in this experiment due to the laboratory conditions which shielded larvae from exposure to predators, pathogens, or parasitoids, as would be expected for wild populations (Moran and Hamilton 1980, Krams et al. 2015). The lengthened development time for larvae reared on diets containing trichomes would potentially increase the exposure of larvae to these environmental pressures and likely increase the difference in survival between treatments.

From the perspective of plants, trichomes, which decreased the amount of food consumed by larvae in this experiment, have the potential to reduce leaf tissue consumption on whole plants. Trichomes may even reduce the number of larvae consuming leaf tissues by increasing larval exposure to predators through slowed larval development time (Moran and Hamilton 1980, Krams et al. 2015). Finally, trichomes have the potential to reduce the number of larvae consuming leaf tissues in the next generation by reducing the number of healthy offspring produced by the current generation (Agrawal 1999). These patterns are similar to those of other, more well-studied plant defenses (Fraenkel 1959, Wittstock and Gershenzon 2002, Gog et al. 2014).

At this point, what remains unclear is the mechanism by which trichomes damage larvae after non-glandular trichomes are consumed by larvae. It is likely to be a combination of factors that may include the fact that trichomes are indigestible and act as a nutritional void; that trichomes may physically plug the gut and slow consumption; or that trichomes may increase metabolic activity in an attempt to digest trichomes or in response to injury from trichomes.

Future studies will need to determine which of these mechanisms are associated with trichomes and the extent to which trichomes affect larval survival in regards to interactions with other species as well as their influence on the next generation.

For now, non-glandular, stellate trichomes should be considered part of the pantheon of plant defenses and accounted for when evaluating the comprehensive defense systems in other plant species. Selecting for increased trichome density while breeding plants could help to reduce pesticide application without reducing value among human consumers. Overall, this study enhances the larger picture of the effects of bodily damage in animals on the short and long-term effects on growth, survival, and reproduction.

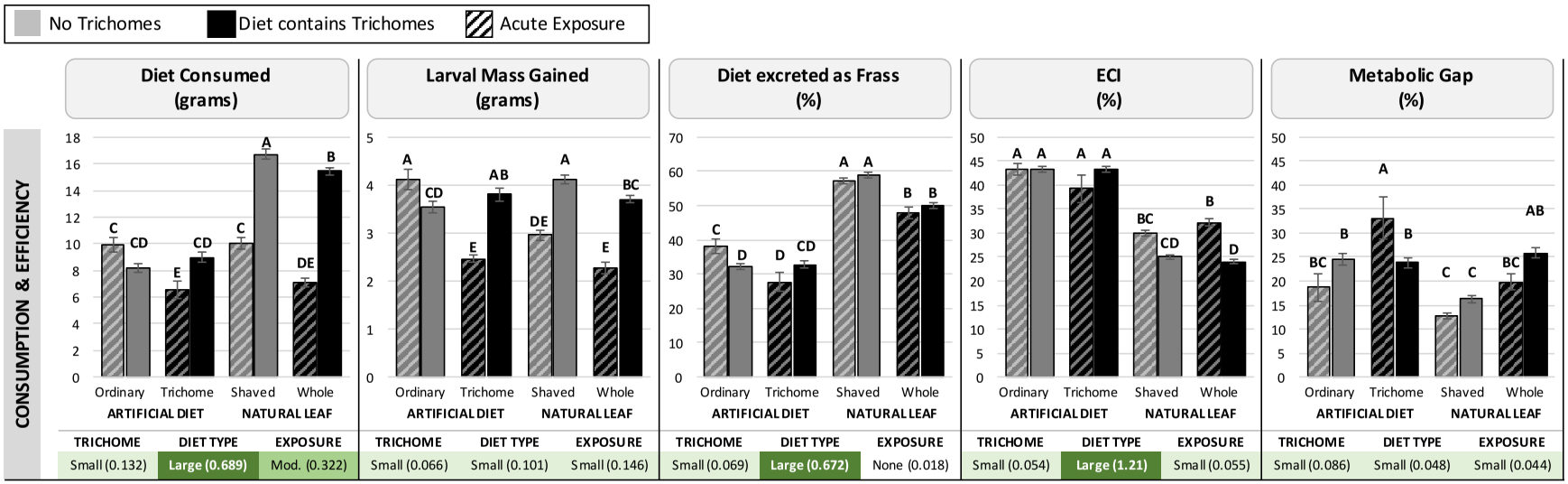

Figure 2-1: Comparison of diet consumption and efficiency metrics for fifth instar hornworm larvae after 48 hours of feeding on treatment diets. Acute larvae were reared on Ordinary Diet from hatching to fifth instar before exposure to treatment diets (n = 110). Chronic larvae were reared on treatment diets from hatching to fifth instar (n = 550). Metrics are organized in columns from left to right: mean amount of diet consumed (g), mean mass gain (g), percent of diet excreted as frass (%), mean efficiency of conversion (ECI, %), and mean metabolic gap (%). Treatments are represented by bars on each graph from left to right: Ordinary Diet, Trichome Diet, Shaved Leaves, and Whole Leaves. Gray bars indicate diets without trichomes; black bars indicate diets with trichomes. Error bars indicate standard error. Different letters on bars indicate statistically significant differences between groups using a Tukey post hoc comparison. Effect sizes for Trichome presence and Natural Diet Base are listed under each bar graph and were calculated using Cohen's f^2 (Small effect: $f^2 > 0.02$, Moderate effect: $f^2 > 0.15$, Large effect: $f^2 > 0.35$) (Cohen 1988).

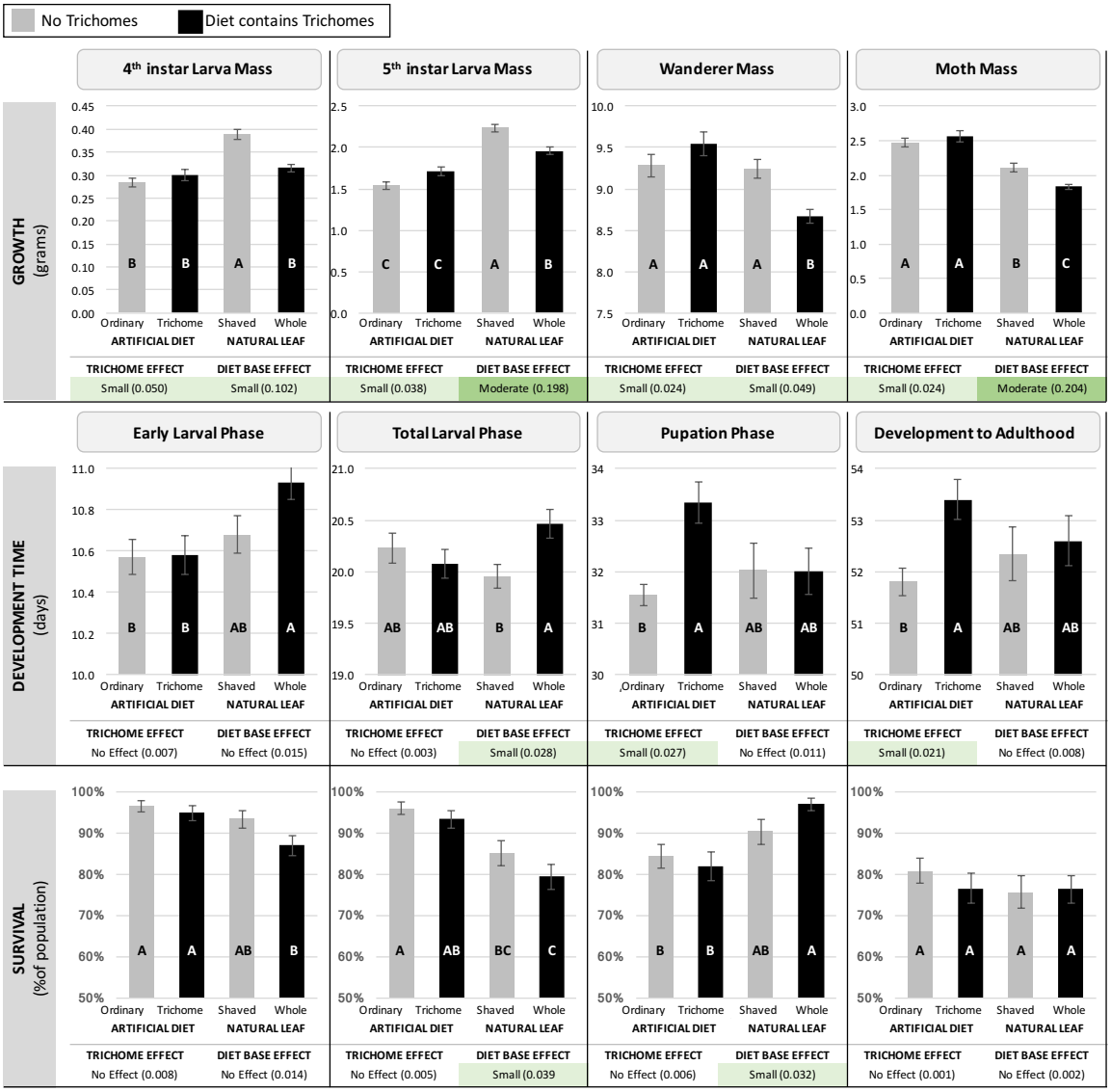

Figure 2-2: Comparison of growth, development time, and survival of hornworms. Mass was measured for larvae at fourth instar (n = 570), fifth instar (n = 550), wandering (n = 521), and after eclosing as moths (n = 460) (Top Row, grams). Development time (Center Row, days) and Survival (Bottom Row, % of population) were calculated for Early Larval Phase (from hatching to fourth instar), Total Larval Phase (from hatching to wandering), Pupation Phase (from wandering to eclosion), Development to Adulthood (from hatching to eclosion) (n = 617). Treatments are represented by bars on each graph from left to right: Ordinary Diet, Trichome Diet, Shaved Leaves, and Whole Leaves. Gray bars indicate diets without trichomes; black bars indicate diets with trichomes. Error bars indicate standard error. Letters on each bar indicate significantly different groups following a Tukey post hoc comparison. Effect sizes for Trichome presence and Natural Diet Base are listed under each bar graph and were calculated using Cohen's f^2 (Small effect: $f^2 > 0.02$, Moderate effect: $f^2 > 0.15$, Large effect: $f^2 > 0.35$) (Cohen 1988).

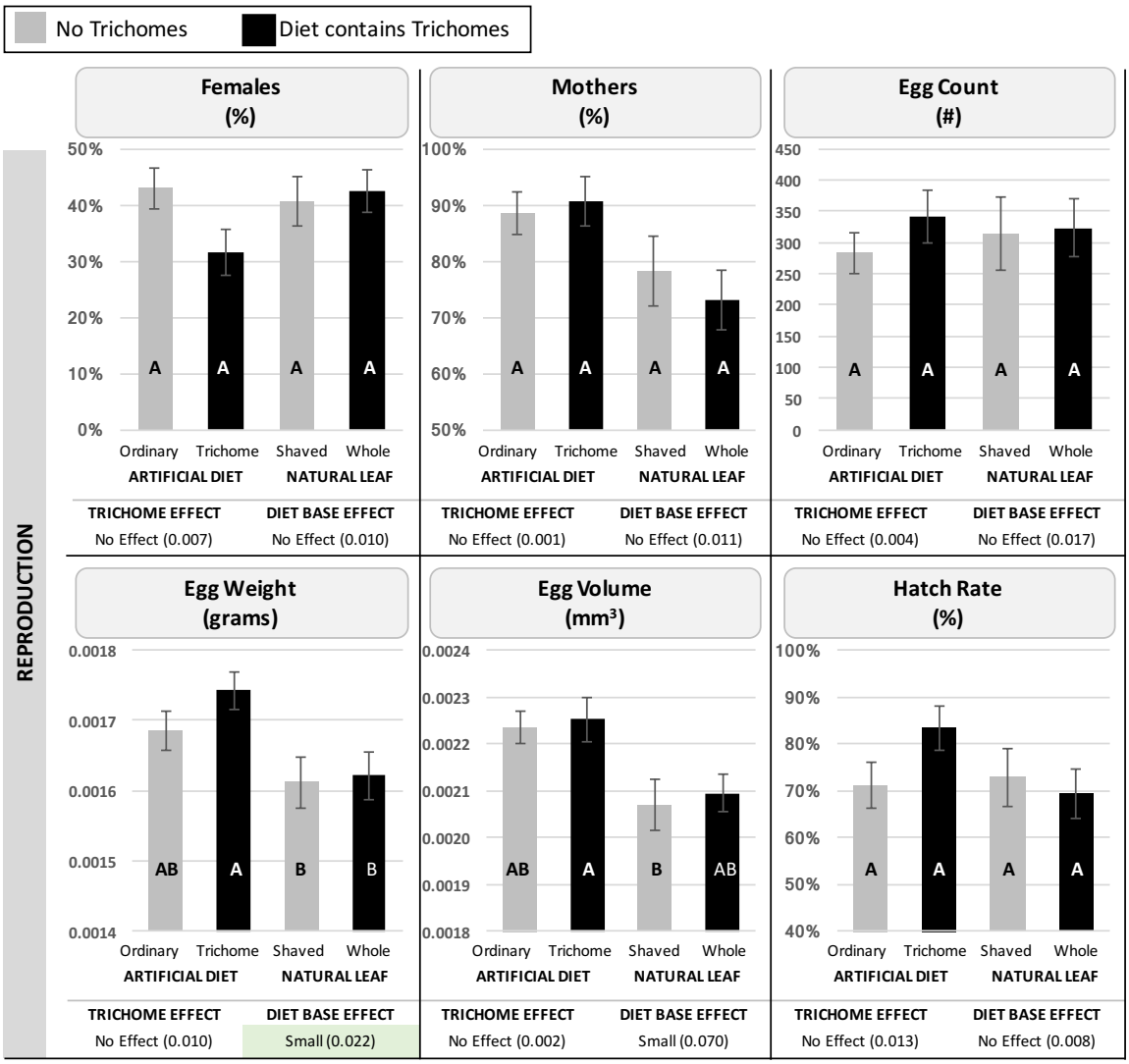

Figure 2-3: Comparison of reproduction metrics for female hawkmoths. Top Row, left-to-right: proportion of the population eclosing as adult females (%), proportion of females laying eggs ("Mothers", %), mean number of eggs laid by mothers (#). Bottom Row, left-to-right: mean mass of a single egg, mean volume of a single egg (mm3), and mean proportion of hatched eggs for individual females (%) (Total number of mothers = 86). Treatments are represented by bars on each graph from left to right: Ordinary Diet, Trichome Diet, Shaved Leaves, and Whole Leaves. Gray bars indicate diets without trichomes; black bars indicate diets with trichomes. Error bars indicate standard error. Letters on each bar indicate significantly different groups following a Tukey post hoc comparison. Effect sizes for Trichome presence and Natural Diet Base are listed under each bar graph and were calculated using Cohen's f^2 (Small effect: $f^2 > 0.02$, Moderate effect: $f^2 > 0.15$, Large effect: $f^2 > 0.35$) (Cohen 1988).

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Chapter 3

Non-glandular trichomes chemically and physically damage herbivores

Abstract

More than impede insect movement, recent studies have shown that non-glandular trichomes directly defend plants by suppressing the growth, survival, and reproduction of small herbivores. Tobacco hornworm larvae (*Manduca sexta*) consuming diets containing trichomes show signs of greater metabolic activity, which may include the upregulation of upregulating tissue repair and immune response genes to repair damage from consuming trichomes. What is not yet understood is whether trichomes affect larvae through nutritional, chemical, or physical mechanisms. To answer this question, this study established a series of treatments that compared intact and manipulated trichomes added to artificial diet to assess the chemical and physical features of stellate, non-glandular trichomes of horsenettle (*Solanum carolinense*). Grinding disrupted the physical shape of trichomes while leaving the chemical composition intact. Cooking disrupted both the trichome shape and chemistry. A final experimental treatment incorporated indigestible, rounded carbon pellets to decrease the nutritional density of the diet, to simulate undigested trichomes, without the consequences of a sharp shape or toxic chemicals. Tobacco hornworm larvae that fed on intact trichomes consumed the least diet, gained the least mass, and were the most metabolically active. Larvae that fed on diet containing ground trichomes did not show quite the same reductions in diet consumption and mass gain or increase in metabolic activity as compared to larvae that fed on diet with intact trichomes, which indicated that manipulating the physical shape of trichomes only partially reversed the harmful effects of trichomes. Larvae that fed on diet containing cooked trichomes or carbon pellets had similar

consumption and metabolic activity to larvae that fed on control diets without any additives, which indicated that manipulating both trichome physical shape and chemistry reversed the harmful effects of intact trichomes and that trichomes did not reduce the nutritional density of diets. Building on these results, the physical impacts of trichomes following consumption were further assessed by tracking the location of diet within larvae using a fluorescent powder. Fluorescent powder was found outside the gut lumen within the hemolymph of larvae reared on diets containing trichomes, but not in the larvae fed on diets without trichomes. This indicated that trichomes puncture the midgut epithelium of larvae allow the contents of the gut to leak into the surrounding hemocoel. Overall, this study found that, following consumption, non-glandular trichomes inhibit larval feeding and growth through chemical and physical mechanisms that disrupt the integrity of the larval gut.

Introduction

Trichomes take many forms and provide many benefits for plants, including water retention, enhanced photosynthesis, and defense from herbivory (Thurston 1970, Gibson 1971, Brewer et al. 1991, Kennedy 2003, Konrad et al. 2015). These small hairs grow directly from the plant epidermis and are broadly classified into two groups (Levin 1973). Glandular trichomes secrete sticky toxins at the distal tip and defend plants by trapping small insects and poisoning herbivores (Thurston 1970, Gibson 1971, Kennedy 2003). Non-glandular trichomes are thought to merely impede the movement of small insects and make it more difficult to bite leaf surfaces (Johnson 1953, Wellso 1973, Pillemer and Tingey 1976, Eisner et al. 1998, Fordyce and Agrawal 2001, Medeiros and Moreira 2002, Mitchell et al. 2016, Kariyat et al. 2017). Unlike toxic chemicals within leaves, trichomes are not thought to have a defensive effect after they are consumed by herbivores. Larvae that consume leaves with higher concentrations of chemical

defense compounds show diminished growth, survivorship, and reproduction (Fox et al. 1995, Pechan et al. 2002, Ojeda-Avila et al. 2003, van Asch et al. 2007, Gog et al. 2014, Portman et al. 2015, Veyrat et al. 2016) due to transcriptional increases for tissue repair and immune response genes to repair damage to the larval midgut (Pechan et al. 2002, Barbeta et al. 2008, Fescemyer et al. 2013).

However, recent work has shown that some non-glandular trichomes can defend plants following consumption by herbivores: herbivorous insect larvae reared on diets containing non-glandular trichomes consumed less diet, were smaller, took longer to reach maturity, and were less likely to survive (Serpi Chapter 2). These negative effects of consuming non-glandular trichomes, much like the effects of consuming chemical defense compounds, are associated with a potential increase in metabolic activity (Serpi Chapter 2). Nutrients that would normally be allocated to growth appear to be requisitioned in response to internal damage caused by the trichomes (Smith and Grodowitz 1983, Herms and Mattson 1992, Rauw 2012, Kariyat et al. 2013).

Damage by non-glandular trichomes may arise from a combination of three features: nutritional, chemical, or physical. Nutritionally, trichomes may act as a void in the diet by occupying space within the larval lumen without contributing metabolically available nutrients, because the trichomes are not digestible and are excreted intact within the frass (Wellso 1973, Kariyat et al. 2017). In this scenario, larvae would need to consume more diet to achieve the same growth as larvae not consuming trichomes (Moran and Hamilton 1980). This might explain the longer development time and the decrease in efficiency converting diet to body mass as previously observed (Serpi Chapter 2).

It may simply be the case that, like chemical compounds consumed in leaf tissue, the chemical compounds of trichomes are able to damage tissues along the larval alimentary canal and trigger the previously observed diversion of energy from growth to other metabolic processes

(Serpi Chapter 2). Trichomes are comprised of leaf epidermal cells that have the same compounds found in leaf tissues, but at lower concentrations due to the additional silica or cellulose that give trichomes their stiff structure (Rodriguez et al. 1984). The previous study used trichomes from horsenettle (*Solanum carolinense*) (Serpi Chapter 2), which is a member of the nightshade family; well known for its toxic chemicals like alkaloids and terpenoids (Bassett and Munro 1986, Cipollini and Levey 1997, Wise 2007, Campbell et al. 2013). If trichomes have enough of the harmful chemical compounds, the differences in larval growth could be from the same tissue damage responses seen in larvae diets with high concentrations of toxins (Pechan et al. 2002, Barbeta et al. 2008, Fescemyer et al. 2013).

Physically the stiff structure and sharp tip of trichomes could internally damage larvae following consumption. Trichomes are indigestible, so their shape is maintained as they pass through the larval digestive tracts and could scrape and poke the larval tissues (Wellso 1973, Kariyat et al. 2017). As with chemical damage, physical tissue damage would account for the potential increase in metabolic activity of the previous study (Serpi Chapter 2). Other studies have used microscopy to reveal that trichomes could catch on the peritrophic matrix of a dissected larva (Kariyat et al. 2017) and possibly pierce the midgut epithelium (Wellso 1973). However, it was unclear if this was affected by the dissection process or if trichomes were fully puncturing the peritrophic membrane *in vivo* and damaging the surrounding epithelium. If trichomes were merely catching on the peritrophic matrix it may slow the progression of the food bolus which would inhibit the ability of the larva to feed. Damage to the peritrophic membrane - a semi-permeable matrix of chitin-microfibrils that encases the large particles of the food bolus while allowing nutrients to pass through to the epithelium where they are absorbed and distributed throughout the body (Gullan and Cranston 2011) - is of little consequence as it is continually shed and rebuilt between instars, when the contents of the gut are expelled prior to molting (Gullan and Cranston 2011). However, if trichomes pass through the peritrophic

membrane and also puncture the epithelium, the damage would have important consequences. The epithelium is a unicellular layer separating the gut lumen from the surrounding hemocoel (Gullan and Cranston 2011) which if punctured, could allow the gut contents to leak into the surrounding open-circulatory system of the insect. The midgut is particularly susceptible as, unlike in the foregut and hindgut, the midgut is not encased in a durable layer of chitin and is thus particularly susceptible to piercing damage (Gullan and Cranston 2011). Damage to the midgut would expose the inner body of the larva to anything consumed by the larva, including toxic plant compounds and potential pathogens (Pechan et al. 2002, Barbeta et al. 2008, Fescemyer et al. 2013) and the combined damage could be worse than either individual mechanism.

To distinguish whether effects of horsenettle (*Solanum carolinense*) trichomes are via chemical, physical or nutritional mechanisms, and to determine if they puncture the midgut epithelium of hornworm larvae (*Manduca sexta*), I performed two experiments. In the first experiment, I wanted to measure separate the nutritional, chemical, and physical effects of trichomes. To do so, I created five treatment diets. Each treatment included an artificial diet base with an additive that isolated a single trichome feature. To test if trichomes act as a nutritional void, indigestible, rounded carbon pellets were added to decrease the nutritional density of the diet, to simulate undigested trichomes, without the consequences of a sharp shape or toxic chemicals. To test physical structure, trichomes were ground into powder to disrupt the physical shape of trichomes while leaving the chemical composition intact. To test the chemistry, trichomes were cooked to disrupt both the trichome shape and chemistry. No distinction was made between beneficial nutritional chemicals and harmful defense chemicals. Two additional treatments, artificial diet with intact trichomes and a control of artificial diet without additives served as comparisons for the manipulated trichome treatments. Following exposure to treatments, I compared the effects the diets on larvae size, diet consumption, frass production, conversion efficiency, and metabolic gap.

In the second experiment, I wanted to determine if trichomes pierced the midgut epithelium and allowed the gut contents to leak into the hemoceol. In order to track the location of diet within the larvae's bodies following consumption, I added fluorescent powder added to two treatment diets which differed in the presence of trichomes (artificial diet with or without trichomes added). If I was able to recover the fluorescent powder in the hemolymph of larvae consuming diet containing trichomes, it would indicate that the integrity of the midgut epithelium was disrupted and allows the gut contents to flow throughout the larval body, rather than remain contained within the alimentary canal. Furthermore, any fluorescent powder that remained in the hemolymph after molting to the fourth instar would also indicate that the larval gut contents had escaped the confines of the gut and been sequestered in the hemocoel where it could not be purged during the regularly emptying of the gut between instars.

Together, these experiments seemed to show that both the chemical and physical characteristics of non-glandular trichomes are responsible for the effects on larvae following consumption. They also revealed that trichome damage punctured the midgut epithelium, allowing the gut contents to leak throughout the larval body.

Methods

Modified Trichomes Experiment

The goal of this experiment was to separate the nutritional, chemical, and physical effects of trichomes using five diet treatments: (1) Ordinary Diet (artificial diet without additives), 2) Nutritional Void (artificial diet with inert carbon pellets to reduce the nutritionally density), 3) Cooked Trichomes (artificial diet with heat-treated trichomes to disrupt both chemical and

physical characteristics), 4) Ground Trichomes (artificial diet with trichomes ground into a powder to remove only the physical shape), and 5) Intact Trichomes (artificial diet with intact trichomes).

The Ordinary Diet was a commercially-available artificial Tobacco Hornworm diet comprised of wheat-germ and agar and prepared according to the manufacturer's directions (P/N: F9783B, Frontier Agricultural Science, Newark, DE). The Nutritional Void treatment tested the effect of nutritional density by creating voids in the diet by adding 1.5 mg of indigestible, rounded carbon pellets (P/N: 329428, Sigma-Aldrich, Darmstadt, Germany) to each gram of artificial diet. This ecologically relevant concentration was determined by identifying concentrations of trichomes that resulted in feeding rates that matched those on natural leaves (see Appendix) and was used for all treatments containing additives. Trichomes for the three trichome-containing treatments were harvested by shattering leaf tissue with palm-sized dry ice pieces and sifting the material through a fine mesh (90 μm). It should be noted that small particles of horsenettle may become airborne during this process, therefore it is highly recommended to wear appropriate personal protective equipment (PPE); including disposable gloves, face mask, and safety goggles. The Cooked Trichomes treatment was prepared by adding trichomes to the boiling water during the preparation of the artificial diet base. While not quantified, I observed that cooking softened trichomes and transformed the physical trichome shape from stiff points into gentle curves that were less likely to pierce and scrape the interior tissues of larvae. It is also likely that cooking altered the chemistry of trichomes, particularly compounds susceptible to heat such as enzymes, possibly rendering compounds less harmful and more easily digestible (Parada and Aguilera 2007). The Ground Trichomes treatment used trichomes ground into a fine powder using a mortar and pestle, and added to the artificial diet base as it cooled, but prior to solidifying. Grinding destroyed the physical shape of the trichomes, while not modifying the chemical content. Finally, the Intact Trichomes treatment added unaltered trichomes to artificial diet base

as it cooled, but prior to solidifying, leaving both the physical shape and chemistry intact. Treatment diets were freshly prepared at the start of each experiment.

Larvae were obtained from my laboratory colony and reared from hatching in groups of ~50 larvae within rearing cups. Cups were assembled by pouring prepared Ordinary Diet (P/N: F9783B, Frontier Agricultural Science Newark, DE) into the base of 11.6 x 15.1 cm deli cups, lining them with a sheet of stiff plastic climbing mesh (P/N: VX620, Frost King by Thermwell, Mahwah, NJ), fitting them with aerated lids (P/N: D32CX, Anchor Packaging, Paragould, AR and P/N: FAB PPLID, Fabri-Kal, Kalamazoo, MI), and inverting them (so that the lid was now the base and the food sat at the ceiling). Larvae were allowed to feed *ad libitum* on Ordinary Diet from hatching to fourth instar.

After molting to fourth instar, 117 larvae were transferred to individual, 100 x 10 mm Petri dishes (P/N: 351029, Corning, Corning, NY) and provided one of the five treatment diets *ad libitum* for 48 hours. Initial larva mass and the mass of diet provided were measured to the nearest 0.001 g using an electronic pan balance. There was no significant difference in the initial mass of larvae assigned to the five different treatments ($F_{4,112} = 0.068$, $p = 0.991$).

After 48 hours of feeding on the respective treatment diet, the mass of the larva, remaining diet, and all frass in the petri dish was measured to the nearest 0.001 g. These values allowed me to estimate larva growth during this period, the amount of diet they consumed, and the relative amount of frass they produced (amount of frass divided by the amount of diet consumed). I also calculated estimates of efficient conversion of diet, and metabolic gap. Conversion efficiency is the amount of ingested food converted to body weight (amount of larval weight gain divided by the amount of diet consumed). Metabolic gap is the proportion of diet consumed that is not accounted for by the amount of larval weight gain and the amount of frass produced (amount of diet consumed minus the amount of larval weight gain and the amount of frass produced; divided by the amount of diet consumed). Together, the metrics for frass,

conversion efficiency, and metabolic gap account for the dispensation of all diet ingested by a larva. Throughout the experiment, conditions within the laboratory were maintained at 16:8 day, 26 ˚C, with ambient humidity.

Fluorescent Diet Experiment

The goal of this experiment was to determine if trichomes physically punctured through both the peritrophic membrane and the epithelium, allowing the gut contents to leak into the hemocoel. To answer this question, only two of the previous treatments were required 1) Ordinary Diet and 2) Intact Trichomes, but with the addition of fluorescent powder to track the movement of the gut contents. Non-water soluble, fluorescent powder (P/N: 65-774, USR Chemicals Inc., Hackettstown, NJ) was added to each treatment diet (5 grams of fluorescent powder per 95 grams of artificial diet). The fluorescent powder was tested for larval consumption and found to be non-toxic and had no affinity for tissues, but slightly reduced the nutritional density of the artificial diet (unpublished data). A 5% mix yielded sufficient fluorescence without compromising larval health or the efficacy of trichomes to affect larval growth (unpublished data). Treatment diets were freshly prepared at the start of each experiment.

Larvae were obtained from my laboratory colony and reared from hatching in groups of ~50 larvae within rearing cups. Cups were assembled by pouring prepared Ordinary Diet (P/N: F9783B, Frontier Agricultural Science Newark, DE) into the base of 11.6 x 15.1 cm deli cups, lining them with a sheet of stiff plastic climbing mesh (P/N: VX620, Frost King by Thermwell, Mahwah, NJ), fitting them with aerated lids (P/N: D32CX, Anchor Packaging, Paragould, AR and P/N: FAB PPLID, Fabri-Kal, Kalamazoo, MI), and inverting them (so that the lid is now the

base and the food sits at the ceiling). Larvae were allowed to feed *ad libitum* on Ordinary Diet prior to experimentation.

After molting to third instar, 48 larvae were transferred from the rearing cups to individual, 100 x 10 mm Petri dishes (P/N: 351029, Corning, Corning, NY). An additional 20 larvae were transferred from the rearing cups to individual Petri dishes after larvae molted to fifth instar. Larvae from both groups were provided one of the two fluorescent treatment *diets ad libitum* for 48 hours. Throughout the experiment, conditions within the laboratory were maintained at 16:8 day, 26 ˚C, with ambient humidity.

Third instar larvae are small enough for fluorescent light to pass through the external epithelium (Fig. 3.1). So, after 48 hours of feeding, I placed third instar larvae on a UV transilluminator (P/N: M-20, UVP, Upland, CA) and photographed them. Larvae were then returned to their respective Petri dishes and continued to feed on assigned treatment diets until molting to fourth instar, after which I photographed them again on the transilluminator. I used ImageJ software (Schneider et al. 2012) to measure the RBG values (red, green, and blue values) of the photographed larvae.

Fifth instar larvae are large enough to easily collect hemolymph samples, so I collected hemolymph from the fifth instar larvae after 48 hours of feeding by clipping the base of the horn and collecting droplets of hemolymph in a micropipette. Immediately, an 8 µL aliquot of hemolymph was placed on the transilluminator and photographed. Again, I used ImageJ software (Schneider et al. 2012) to measure the RBG values of the photographed hemolymph.

Statistical Analyses

Statistical analyses were performed in R (R Core Team 2014). The effects of the five treatments used in the Modified Trichomes Experiment were tested with separate Analysis of Covariance models for each larval measure (the amount of diet consumed, larval mass gain

(change in mass over 48 hours calculated by subtracting the initial mass from the final mass), frass production, conversion efficiency, and metabolic gap) using the '*aov*' function of the base package and the post hoc comparisons were performed using the '*HSD.test*' function from the agricolae package (de Mendiburu 2017). All models included treatment as a factor and included initial larval mass as a covariate to account for size-dependent effects (e.g., larger animals should consume more diet and produce more frass than smaller animals, etc.), but omitting mass did not qualitatively change the results.

The color of third and fourth instar larvae from the Fluorescent Diet Experiment, after feeding on treatment diets for 48 hours, was analyzed using separate t-tests to compare the red, green, and blue color levels between treatments using the '*t.test*' function of the base package in R (R Core Team 2014) and the post-hoc '*HSD.test*' function from the agricolae package (de Mendiburu 2017). The t-tests were repeated to analyze larvae color after molting to fourth instar.

The color of fifth instar hemolymph collected from larvae after feeding on treatment diets for 48 hours was analyzed using separate linear regression models for red, green, and blue color levels using the 'lm' function of the base package in R (R Core Team 2014) and the post-hoc '*HSD.test*' function from the agricolae package (de Mendiburu 2017). Each model included treatment, and the bleed order of each larvae in which hemolymph aliquots were collected and photographed to account for influences of fluctuations in light in the surrounding environment.

Results

Modified Trichomes Experiment

Diet consumption

Treatment diet significantly affected the amount of diet larvae consumed ($F_{4,111} = 16.80$, $p < 0.001$; Fig. 3.2). Larvae that consumed the Nutritional Void diet ate significantly more than larvae that consumed the Cooked Trichome Diet and the Intact Trichome Diet. Larvae that consumed the Ordinary Diet and the Ground Trichome Diet consumed an intermediate amount that was significantly more than larvae that consumed the Intact Trichome Diet, but similar to the amount consumed by larvae eating the Nutritional Void and Cooked Trichome Diet. Larger larvae consumed more diet than smaller larvae ($F_{1,111} = 255.36$, $p < 0.001$).

Larval mass gained

Treatment diet significantly affected the amount of mass larvae gained in the 48 hours of the experiment ($F_{4,111} = 11.57$, $p < 0.001$; Fig. 3.2). Larvae that consumed the Ordinary Diet, Nutritional Void Diet, Cooked Trichomes Diet, and the Ground Trichomes Diet all gained similar amounts of mass; whereas larvae that consumed the Intact Trichomes Diet gained significantly least mass and were significantly smaller than larvae consuming the other diets. Larger larvae gained more mass than smaller larvae ($F_{1,111} = 651.96$, $p < 0.001$).

Diet excreted as frass

Treatment diet significantly affected the proportion of diet excreted as frass in the 48 hours of the experiment ($F_{4,111} = 8.43$, $p < 0.001$; Fig. 3.2). Larvae that consumed the Ordinary Diet, the Cooked Trichomes Diet, and the Nutritional Void Diet produced similar amounts of frass. Larvae that consumed the Intact Trichomes Diet produced significantly less frass than did larvae that consumed the Ordinary Diet and the Cooked Trichomes Diet, but similar amounts of frass to consuming the Nutritional Void Diet and the Ground Trichomes Diet. Larvae consuming the Ground Trichomes Diet produced significantly less frass than larvae consuming the Ordinary Diet. Larger larvae more frass than smaller larvae ($F_{1,111} = 48.67$, $p < 0.001$).

Conversion efficiency

Treatment diet significantly affected the proportion of consumed diet that was converted to body mass ($F_{4,111} = 3.52$, $p = 0.010$; Fig. 3.2). Efficiency of larvae that consumed the Cooked Trichomes Diet was significantly greater than larvae that consumed the Intact Trichomes Diet. Larvae that consumed the Ordinary Diet, Nutritional Void Diet, and Ground Trichomes Diet had intermediate efficiencies that were not significantly different from each other or larvae that consumed the Cooked Trichomes Diet or the Intact Trichomes Diet. Larger larvae had higher conversion efficiency than smaller larvae ($F_{1,111} = 118.11$, $p < 0.001$).

Metabolic gap

Treatment diet significantly affected the proportion of consumed diet that was used in unknown metabolic processes ($F_{4,111} = 6.56$, $p < 0.001$; Fig. 3.2). Larvae that consumed the Intact Trichomes Diet had metabolic gaps that were significantly greater than larvae that consumed the Ordinary Diet, Nutritional Void Diet, and Cooked Trichomes Diet. Larvae that consumed the Ground Trichomes Diet had intermediate metabolic gaps and were not significantly different from larvae in any other treatment. Larger larvae had smaller metabolic gaps than smaller larvae ($F_{1,111} = 114.68$, $p < 0.001$).

Fluorescent Diet Experiment

Third Instar Larvae Color After Feeding for 48 hours

Third instar larvae color was assessed after feeding on treatment diets for 48 hours and before their guts were voided during molting. Larvae that consumed Fluorescent Intact Trichome

Diet had higher Red values ($t_{49.97}$ = -5.68, $p < 0.001$), similar Green values ($t_{46.34}$ = -0.17, $p = 0.866$), and lower Blue values ($t_{54.80}$ = 4.81, $p < 0.001$) than larvae that consumed Fluorescent Ordinary Diet (Fig. 3.3).

Larvae Color After Molting to Fourth Instar

Fourth instar larvae color was assessed after larvae had molted to fourth instar and before feeding had resumed. Larvae that consumed Fluorescent Intact Trichome Diet had similar Red values ($t_{42.61}$ = 1.25, $p = 0.217$) and Green values ($t_{44.26}$ = -1.31, $p = 0.198$), and higher Blue values ($t_{41.34}$ = -2.33, $p = 0.025$) than larvae that consumed Fluorescent Ordinary Diet (Fig. 3.3).

Within Treatment Comparisons of Larvae Color Before and After Molting

Larval body color was compared over time for larvae feeding on the Fluorescent Intact Trichome Diet after feeding for 48 hours and again after the larvae had molted to fourth instar. Larvae had significantly lower Red values ($t_{44.57}$ = 13.56, $p < 0.001$) and Green values ($t_{44.38}$ = 5.26, $p < 0.001$) after molting to fourth instar. However, Fluorescent Intact Trichome Diet retained the same Blue value after molting to fourth instar ($t_{44.39}$ = -0.77, $p = 0.446$; Fig. 3.4).

The same tests were repeated for larvae feeding on the Fluorescent Ordinary Diet, but all three of the color values were significantly different after molting (Red: $t_{44.19}$ = 5.24, $p < 0.001$; Green: $t_{42.20}$ = 7.09, $p < 0.001$; Blue: $t_{38.50}$ = 5.54, $p < 0.001$; Fig. 3.4).

Fifth Instar Hemolymph Color After Feeding for 48 hours

Hemolymph collected from fifth instar larvae after feeding on treatment diets for 48 hours and prior to wandering behavior. Larvae that consumed Fluorescent Intact Trichome Diet had similar Red values ($t_{2,17}$ = -0.34, $p = 0.742$) and Green values ($t^{2,17}$ = 0.02, $p = 0.981$), and higher Blue values (t2,17 = 2.23, $p = 0.040$) than larvae that consumed Fluorescent Ordinary Diet

(Fig. 3.5). Bleed order did not affect Red values ($t_{2,17}$ = -1.55, p = 0.139) and Green values ($t_{2,17}$ = -1.51, p = 0.149), but larvae that were bled later in the experiment had lower Blue values ($t_{2,17}$ = -2.77, p = 0.013).

Discussion

The results of this study indicate that trichomes used both physical and chemical methods to inhibit larval growth and did not reduce the nutritional density of diet. When larvae consumed intact trichomes added to artificial diet, they consumed less diet, gained less mass, and produced less frass than peers that consumed artificial diet without trichomes. These larvae also seemed to divert dietary resources from growth to other energy-intensive processes with lower conversion efficiencies and had greater metabolic gaps. These results align with previous observations of effects on larvae that consumed diets with and without trichomes, including when dietary trichomes were intact on natural leaf surfaces (Smith and Grodowitz 1983, Serpi Chapter 2). Larvae were also fed a variety of diets that manipulated the nutritional, chemical, and physical characteristics of trichomes to distinguish which characteristics led to these effects.

Trichome indigestibility, and therefore nutritional unavailability, is unlikely to be the trichome feature that harms larvae and protects the plant by reducing diet consumption. Diet consumption and efficiency metrics did not differ between larvae that fed on the treatment diet mimicking a nutritional void (Nutritional Void Diet) and larvae that fed on the control diet without additives (Ordinary Diet). However, larvae that fed on the Nutritional Void Diet consumed significantly more diet, gained significantly more mass, and had significantly smaller metabolic gaps than larvae that fed on diet containing intact trichomes (Intact Trichomes Diet). This indicates that reduced nutritional density is not the characteristic that causes the effects observed after larvae consume trichomes. If nutritional density was culpable, the effects of

consuming Nutritional Void Diet would match the small size and increased metabolic gap of larvae consuming Intact Trichomes observed in this study and other studies of nutritional density (Moran and Hamilton 1980, Parada and Aguilera 2007, Kariyat et al. 2017).

Trichome chemistry was found to be partially culpable for the effects of trichomes on larvae. Diet consumption and efficiency metrics did not differ between larvae that fed on the treatment diet that contained trichomes ground into a fine powder to remove their physical shape without altering their chemical content (Ground Trichomes Diet) and larvae that fed on Ordinary Diet, except that larvae that fed on Ground Trichomes Diet produced less frass. This indicates chemistry and not physical shape may be responsible for the constipating effect of trichomes. Larvae that fed on Ground Trichomes Diet consumed more diet than larvae that fed on Intact Trichomes Diet which indicates that the trichomes physical shape, and not their chemistry, may inhibit larval feeding. The additional diet larvae consumed seems to have been allocated to growth rather than other metabolic processes. Larvae that fed on Ground Trichomes Diet gained more weight than larvae that fed on Intact Trichomes Diet, but their metabolic activity did not significantly differ from larvae that fed on Intact Trichomes Diet. However, the metabolic activity of larvae that fed on Ground Trichomes also did not significantly differ from larvae that fed on Ordinary Diet, despite the significant difference in metabolic activity between larvae that fed on Ordinary Diet and Intact Trichomes Diet. The mean metabolic gap for larvae that fed on Ground Trichomes Diet fell between the lower mean for larvae that fed on Ordinary Diet and the higher mean for larvae that fed on Intact Trichomes Diet. This seems to indicate that trichome chemistry alone was partially responsible for some of the effects of consuming trichomes, but was not enough to the match the full-strength effects of consuming intact trichomes which retained both their chemistry and physical shape. It may be that trichomes deliver both the effects of plant

defense chemicals (Fox et al. 1995, Pechan et al. 2002, Ojeda-Avila et al. 2003, van Asch et al. 2007, Gog et al. 2014, Portman et al. 2015, Veyrat et al. 2016) and the effects of physical plant defenses (Smith and Grodowitz 1983, Bassett and Munro 1986, Milewski et al. 1991, Gowda 1996, Obeso 1997, Hanley et al. 2007).

The physical shape of trichomes was again found to be partially culpable for the effects of trichomes on larvae after the trichomes were cooked. Cooking softened trichomes and altered their shape from sharp points to soft curves. Cooking is also likely to have altered the chemical content of trichomes which could reduce the effect of harmful chemicals and unlock previously unavailable nutrients (Parada and Aguilera 2007). Diet consumption and efficiency metrics did not differ between larvae that fed on the treatment diet that contained cooked trichomes (Cooked Trichomes Diet) and larvae that fed on Ordinary Diet. However, larvae that fed on Cooked Trichomes Diet differed from larvae that fed on Intact Trichomes Diet at every metric and showed increased diet consumption, increased weight gain, increased frass production, more efficient conversion, and smaller metabolic gaps. Therefore, it appears that the cooking process eliminated the harmful features of trichomes and that cooked trichomes were rendered harmless and had the same effect on larvae as the artificial diet control, Ordinary Diet.

It is interesting that cooking trichomes removed the constipating effect seen following the consumption of the Intact Trichome Diet or the Ground Trichome Diet that removed the physical shape of trichomes while retaining the chemical content. It was hypothesized that the physical shape of trichomes would have caused blockages along the alimentary canal (Wellso 1973). Unfortunately, these results only show that trichome chemistry contributes to larval constipation, but it is unclear if their physical shape also plays a role. To assess the role of trichome shape in frass production, a future study would need to establish a treatment with artificial trichomes that retain the trichome shape, but not the trichome chemistry, as this study failed to find a suitable trichome substitute.

The physical damage dealt by trichomes was further explored by using fluorescent powder to track the location of diet within the larval body. In larvae that fed on artificial diet without trichomes, fluorescent powder was only visible along the alimentary canal. In contrast, in larvae that fed on artificial diet with intact trichomes, fluorescent powder was visible throughout the larval body, except within head cavities. This indicated that the fluorescent powder had escaped the confines of the midgut epithelium, likely along with the rest of the non-fluorescent contents of the gut. Fluorescent powder in the surrounding hemocoel could not be excreted within the frass exiting the alimentary canal. Therefore, larvae that fed on diet containing trichomes accumulated more fluorescent powder and were significantly brighter than larvae that had not consumed trichomes.

Following molting to fourth instar, the fluorescent powder in the hemocoels of larvae that had fed on diet containing trichomes would not be eliminated when the larvae emptied their guts and shed their peritrophic membrane along with their outer epithelium. This was confirmed when larvae that had fed on diet containing trichomes retained significantly more Blue value after molting than larvae feeding on diet without trichomes. Furthermore, among the larvae feeding on diet containing trichomes, the Blue value was not significantly different at third instar during feeding and at fourth instar after molting. In contrast, larvae feeding on diet without trichomes were significantly darker after molting. This indicates that in the presence of trichomes, some of the fluorescent powder and other gut contents entered the hemocoel prior to the larvae emptying their guts for molting. However, larvae that did not consume trichomes did not appear to suffer punctures in the midgut epithelium, and when they molted, they were able to eliminate all of the fluorescent powder they had previously consumed.

In corroboration with these observations, color measurements of the hemolymph extracted from fifth instar larvae also suggested trichomes damaged the midgut epithelium; allowing the fluorescent powder and other gut contents and to escape into the hemocoel.

Hemolymph collected from larvae feeding on diet containing trichomes was significantly brighter than hemolymph from larvae feeding on diet without trichomes. While this difference was easy to detect with the naked eye, digitally distinguishing the colors was more difficult and was influenced by light contamination. This is because hemolymph is translucent, mostly colorless with a slight blue sheen, and easily influenced by light in the surrounding environment (personal observation). Future studies could reduce light contamination through the use of a dark room. Despite these limitations, a hemolymph color difference was detected and the difference underscored the third instar results which indicated that trichomes puncture the midgut epithelium.

Together all of these results suggest that, following consumption by larvae, trichomes pose both a chemical and physical threat, without reducing nutritional density. Trichomes punctured both the peritrophic membrane and the midgut epithelium which allowed fluorescent powder and the other gut contents to escape to the interior of the larval body. In addition to damaging tissues, this could expose the previously protected hemocoel to toxic leaf compounds and pathogens consumed in the diet (Pechan et al. 2002, Barbeta et al. 2008, Fescemyer et al. 2013) and is expected to initiate an upregulation of tissue repair mechanisms and immune response proteins (Fescemyer et al. 2013). In turn, this would explain the increase in metabolic gap and decrease in conversion efficiency for larvae feeding on diets containing trichomes (Smith and Grodowitz 1983, Serpi Chapter 2). Ultimately, understanding these chemical and physical mechanisms enhances our understanding of the role of non-glandular trichomes within the broader picture of plant defense.

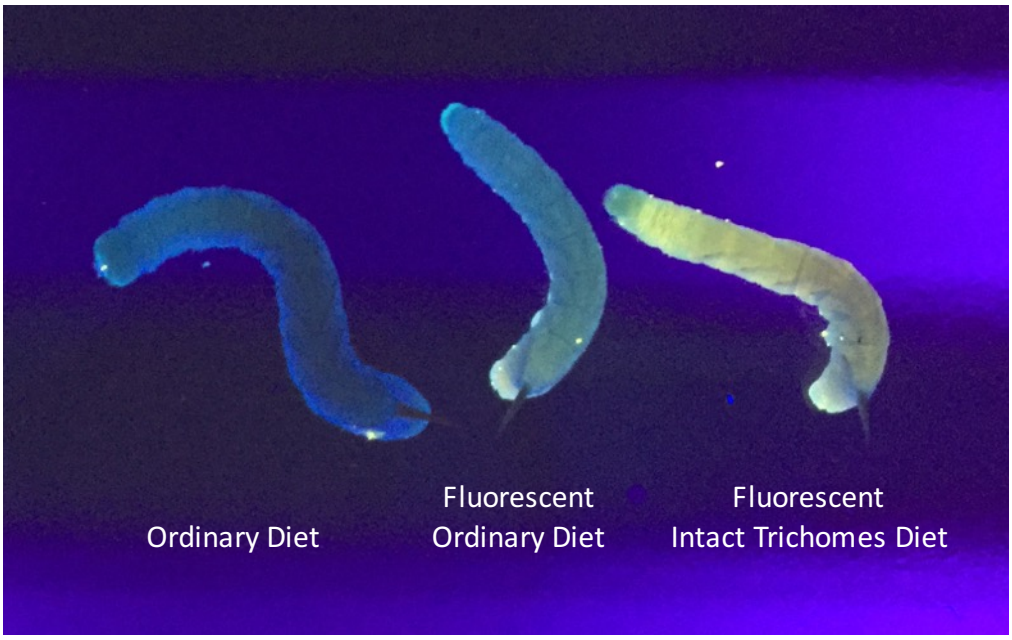

Figure 3-1: Image of larvae following consumption of Fluorescent Diet Experiment treatments. Larvae that had consumed Ordinary Diet show no natural fluorescence. Larvae that consumed Fluorescent Ordinary Diet only showed fluorescence along the alimentary canal. Larvae that consumed Fluorescent Intact Trichomes Diet showed fluorescence throughout the hemocoel.

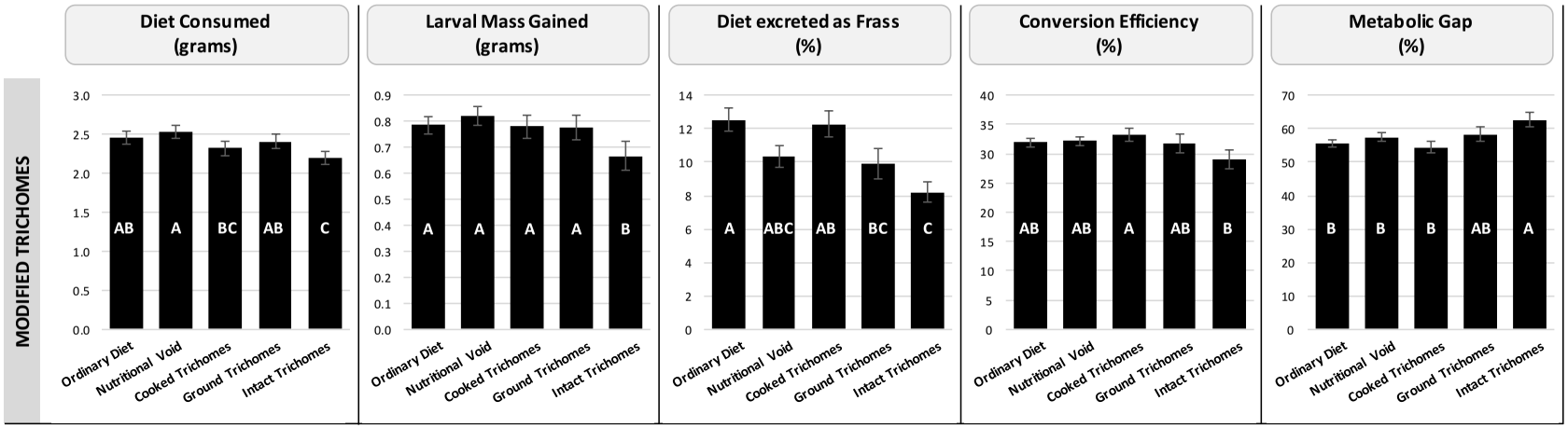

Figure 3-2: Modified Trichomes Experiment: Comparison of diet consumption and efficiency metrics for fourth instar hornworm larvae after 48 hours of feeding on treatment diets (n = 117). Larvae were reared on Ordinary Diet from hatching to fourth instar before exposure to treatment diets. Metrics are organized in columns from left to right: mean amount of diet consumed (g), mean mass gain (g), percent of diet excreted as frass (%), mean efficiency of conversion (%), and mean metabolic gap (%). Larvae were fed an artificial diet without additives (Ordinary Diet), or artificial diet with inert carbon pellets (Nutritional Void), or artificial diet with heat-treated trichomes (Cooked Trichomes), or artificial diet with trichomes ground into a powder (Ground Trichomes), or artificial diet with intact trichomes (Intact Trichomes). Error bars indicate standard error. Different letters on bars indicate statistically significant differences between groups using a Tukey post hoc comparison.

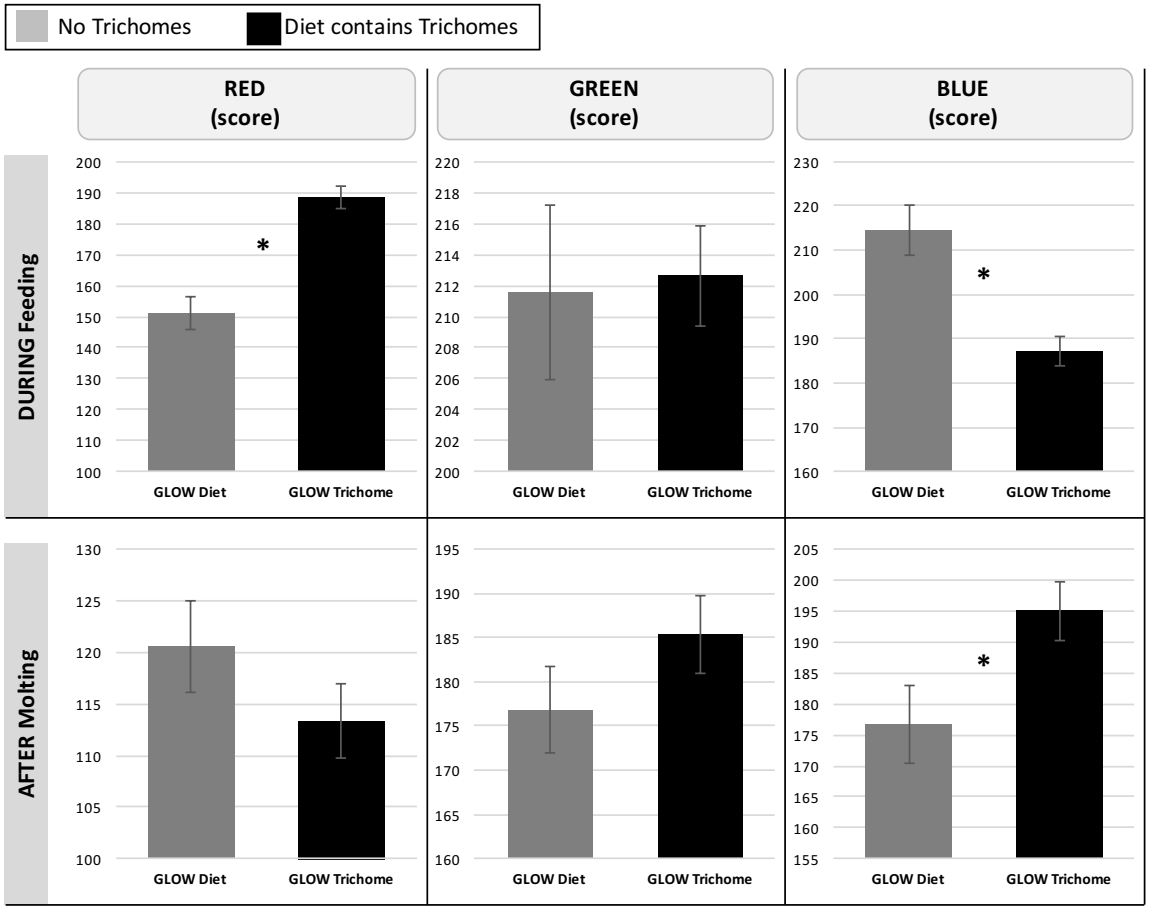

Figure 3-3: Fluorescent Diet Experiment: Comparison of larvae color for third instar hornworm larvae after 48 hours of feeding on treatment diets and again after molting to fourth instar (n = 48). Color values were measured in Image J using the scale from 0 (black) to 255 (white) for Red, Green, and Blue values. Larvae were fed artificial diet with fluorescent powder (Fluorescent Ordinary Diet) or artificial diet with trichomes and fluorescent powder (Fluorescent Intact Trichomes Diet). Error bars indicate standard error. Asterisks between bars indicate statistically significant differences between groups.

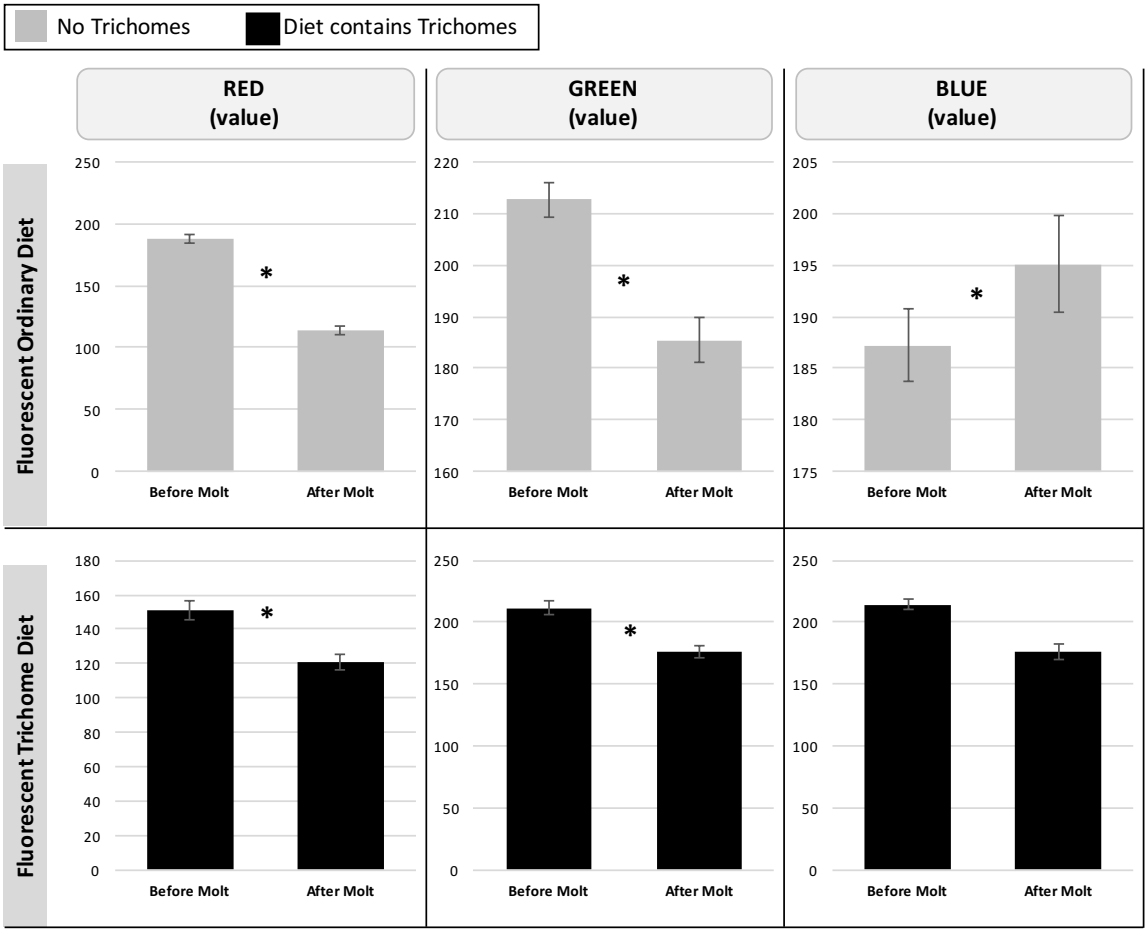

Figure 3-**4**: Fluorescent Diet Experiment: Comparison of larvae color within treatments before and after molting to fourth instar (n = 48). Color values were measured in Image J using the scale from 0 (black) to 255 (white) for Red, Green, and Blue values. Larvae were fed artificial diet with fluorescent powder (Fluorescent Ordinary Diet) or artificial diet with trichomes and fluorescent powder (Fluorescent Intact Trichomes Diet). Error bars indicate standard error. Asterisks between bars indicate statistically significant differences between groups.

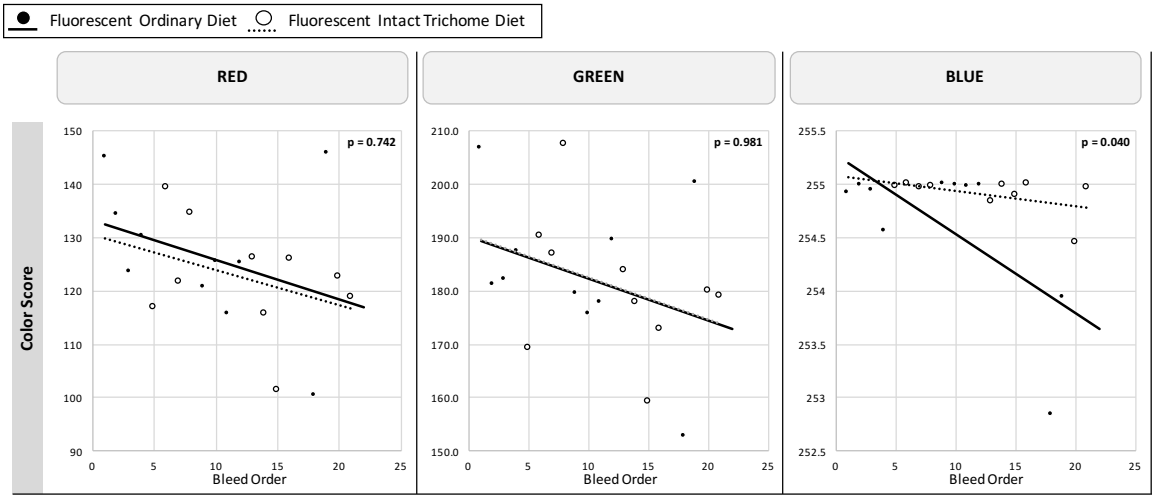

Figure 3-**5**: Fluorescent Diet Experiment: Comparison of hemolymph color for fifth instar hornworm larvae after 48 hours of feeding on treatment diets (n = 20). Color values were measured in Image J using the scale from 0 (black) to 255 (white) for Red, Green, and Blue values. Bleed order indicate the order in which hemolymph aliquots were collected and photographed. Closed black circles and solid lines represent larvae that were fed artificial diet with fluorescent powder (Fluorescent Ordinary Diet). Open circles and dotted lines represent larvae that were fed artificial diet with trichomes and fluorescent powder (Fluorescent Intact Trichomes Diet).

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Chapter 4

Damage from non-glandular trichomes has transgenerational consequences for an herbivore

Abstract

Organisms are constantly buffeted by the harsh realities of their environment. Some organisms have evolved mechanisms to prepare their offspring to face similar environments. Through parental effects, parents can enrich offspring with quality nutrition and share epigenetic modifications. One example of an environmental pressure that is able to induce transgenerational effects is organismal damage from consuming plant defenses. When consuming plant-based diets, herbivores face challenges such as low nutrition density, toxic defense chemicals, and sharp structures. While the ability of nutritional quality and chemical defenses to trigger transgenerational effects has been studied, the transgenerational effects of physical plant defenses is relatively unknown, in part due to the difficulties of adequately separating physical structures from the leaf chemistry. This study examined the transgenerational effects of non-glandular trichomes on the offspring of tobacco hornworms (*Manduca sexta*) reared on artificial and natural leaf diets with or without the defensive trichomes of horsenettle (*Solanum carolinense*). Offspring were reared on either a natural leaf diet or an artificial diet lacking any plant defenses. Offspring of parents reared on trichome-containing diets were significantly smaller and tended to be darker, consumed a greater amount of diet, had differences in body mass gain and other metabolic processes, and had increased survival, than offspring of parents never exposed to dietary trichomes. These parental effects held, regardless of which diet the offspring themselves consumed. Overall, trichomes consumed by hornworms affect subsequent offspring. Internal damage caused by diets may reduce an organism's quality of life, but their offspring appear to be less susceptible to effects of such damage.

Introduction

Organismal damage occurs in many forms. Microbial infections, external wounds, and dietary toxins are among many examples of physical harm which can decrease an organism's quality of life. Beyond their own lifetime, damage may result in transgenerational effects for an organism's offspring. In some cases, organisms suffering from a poor quality of life will produce fewer or smaller offspring (Roach and Wulff 1987, Mousseau and Dingle 1991). In other cases, offspring may be born ready to face similar environmental challenges (Agrawal et al. 1999, Freitak et al. 2014, McCormick et al. 2019). Environmental effects experienced by the parental generation that lead to transgenerational effects on offspring are typically referred to as maternal effects (Roach and Wulff 1987, Mousseau and Dingle 1991). The mechanisms of maternal effects can be divided into four categories: genetic, provisioning, phenotypic, and epigenetic (Roach and Wulff 1987, Mousseau and Dingle 1991, Freitak et al. 2014). Traditionally these mechanisms were collectively called maternal effects because it was thought that fathers were only able to contribute their nuclear genes to offspring. However, the term "parental effects" may be more appropriate given more recent studies that have shown fathers can also affect their offspring through provisioning and epigenetic inheritance (Dussourd et al. 1988, Freitak et al. 2009, Triggs and Knell 2012, Love et al. 2013, Dew-Budd et al. 2016).

Damage-induced parental effects are well studied in plants because they are simple to clone, easy to selectively mate, and ethically unambiguous to damage. Upon wounding, plants increase their defense system to reduce subsequent herbivory, and these elevated defense levels can be passed on to their offspring (Fox et al. 1995, Pechan et al. 2002, van Asch et al. 2007, 2010, Raguso et al. 2007, Gog et al. 2014, Portman et al. 2015a, Veyrat et al. 2016). These same plant defense systems can be used as a natural source of damage to study damage-induced parental effects in herbivorous animals. In addition to acquiring nutrients, herbivores consuming

plant-based diets must also contend with harmful chemical plant defenses. Plants contain toxic compounds, such as alkaloids, (Fraenkel 1959, Cipollini and Levey 1997, Wittstock and Gershenzon 2002, Fürstenberg-Hägg et al. 2013), that internally damage herbivores following consumption (Pechan et al. 2002, Barbeta et al. 2008, Fescemyer et al. 2013). Herbivores that consume these plants show diminished growth, survivorship, and reproduction (Fox et al. 1995, Pechan et al. 2002, van Asch et al. 2007, Raguso et al. 2007, Gog et al. 2014, Portman et al. 2015, Veyrat et al. 2016). However, the same chemical defenses can elicit parental effects that benefit the subsequent generation. Offspring of parents exposed to plant chemical defenses tend to be larger, develop more quickly, and are more likely to survive than offspring on non-exposed parents (Rossiter 1991a, 1991b, Fox et al. 1995, Awmack and Leather 2002, van Asch et al. 2007).

However, chemical defenses are only part of a plant's arsenal: plants also have physical defenses that include traits such as leaf toughness (Turner 1994, Hanley et al. 2007), sharp spines (Milewski et al. 1991, Gowda 1996, Obeso 1997), and trichomes (Britton and Brown 1913, Thurston 1970, Kennedy 2003). Leaf toughness (which increases the difficulty chewing/piercing leaves and reduces nutritional density) and sharp spines are known to hamper herbivore diet consumption and growth (Turner 1994, Gowda 1996, Obeso 1997, Hanley et al. 2007). Trichomes are small hairs, typically only a few millimeters tall, extending from the epidermis of the plant and benign to large herbivores. They are most commonly associated with water retention and inhibiting insect movement (Johnson 1953, Levin 1973, Brewer et al. 1991, Fordyce and Agrawal 2001, Medeiros and Moreira 2002, Konrad et al. 2015, Mitchell et al. 2016). However, recent work has shown that consuming stellate, non-glandular trichomes of horsenettle (*Solanum carolinense*) led to detrimental effects, similar to those of chemical defenses (Fox et al. 1995, Pechan et al. 2002, van Asch et al. 2007, Raguso et al. 2007, Gog et al. 2014, Portman et al. 2015, Veyrat et al. 2016), on the growth, survival, and reproduction of tobacco

hornworms (*Manduca sexta*) (Serpi Chapter 2). These detrimental effects are associated with an increase in metabolic activity which divert resources from growth and reproduction. It is likely that trichomes, which are not digested within the larvae and are excreted intact within the frass, scrape and puncture the midgut epithelium. Such internal damage is akin to external damage and wounding in other species, which has been shown to induce parental effects that prepare plant and animal offspring for similar physical damage (Agrawal et al. 1999, McCormick et al. 2019). As with the consumption of chemical defenses, consuming physical defenses, like trichomes, could trigger parental effects by reducing the available resources for egg provisioning or through epigenetic modifications of gene transcription (Herms and Mattson 1992, Rauw 2012, Kariyat et al. 2013). Indeed, recent work has suggested that eggs of females reared on diets containing trichomes tended to be larger and heavier than eggs of other females; traits which are generally associated with faster development and larger offspring (Rossiter 1991b, Mousseau 1998, Cahenzli and Erhardt 2013). Furthermore, the color of the eggs changed based on the content of the maternal diet and may indicate a difference in egg provisioning (Fox et al. 1995), Serpi Chapter 2). Together, these size and color discrepancies could indicate that trichomes are enough of an environmental pressure to trigger parental effects that represent a transgenerational injury or adaptive response that prepares the next generation to face similar conditions.

In this study, I sought to assess the transgenerational effects of consuming non-glandular trichomes for a lepidoptera species (*Manduca sexta*). Few studies have followed the transgenerational effects through offspring maturation to adulthood (Cahenzli and Erhardt 2013, Woestmann and Saastamoinen 2016), therefore I elected to monitoring offspring from hatching to sexual maturity. I established the parental generation by developing four dietary treatments. To examine the effects of *in situ* trichomes, I used natural leaves of a single species of horsenettle (*Solanum carolinense*) and physically removed trichomes or left them intact to establish natural leaf treatments with and without trichomes. I also included a pair of artificial diet treatments, one

with and one without added trichomes, to omit the effects of leaf chemistry. Upon reaching maturity, female hawkmoths were mated with males reared on the same treatment diet (1: natural leaves with removed trichomes, 2: natural leaves with trichomes, 3: artificial diet without trichomes, 4: artificial diet with added trichomes).

I also sought to distinguish the effects of maternal and paternal diet consumption on offspring. To answer this question, I used the same dietary treatments in the parental generation. However, instead of simply mating females and males from the same treatment, I developed a reciprocal cross design by mating females to males reared on different treatments (1: both parents fed artificial diet without trichomes, 2: both parents fed natural leaves with trichomes, 3: mother fed artificial diet without trichomes and father fed natural leaves with trichomes, 4: mother fed natural leaves with trichomes and father fed artificial diet without trichomes).

Offspring from both mating schemes were reared on artificial diet without trichomes or natural leaves with trichomes and monitored from hatching to sexual maturity. I expected that, like chemical plant defenses, if non-glandular, stellate trichomes damage was able to trigger parental effects, they should have a net positive effect on the offspring, regardless of the offspring's diet. Specifically, offspring of parents reared on diet containing trichomes would consume more diet, have a larger body mass, reach sexual maturity more quickly, and be more likely to survive to adulthood. Additionally, if paternal diet was found to affect offspring phenotype, it would be likely that epigenetic modifications were the mechanism behind the parental effects.

Methods

Larvae were obtained from my laboratory colony and were reared on one of four treatment diets: 1) Ordinary Diet (artificial diet without trichomes added), 2) Trichome Diet

(artificial diet with trichomes added), 3) Shaved Leaves (natural leaves with most trichomes physically removed), and 4) Whole Leaves (natural leaves with trichomes unmodified). The Ordinary Diet was a commercially-available artificial Tobacco Hornworm diet comprised of wheat-germ and agar and prepared according to the manufacturer's directions (P/N: F9783B, Frontier Agricultural Science, Newark, DE). The Trichome Diet was prepared by adding 1.5 mg of trichomes to each gram of Ordinary Diet; this ecologically relevant concentration was determined by identifying concentrations of trichomes that resulted in feeding rates that matched those on natural leaves (see Appendix). Trichomes were harvested by combining leaf tissue and palm-sized dry ice pieces within a cloth bag and shattering this tissue by shaking the bag. The resulting material was shifted through a fine mesh (90 μm) to separate the trichomes from leaf particles and dry ice. It should be noted that small particles of horsenettle may become airborne during this process, therefore it is highly recommended to wear appropriate personal protective equipment (PPE); including disposable gloves, face mask, and safety goggles. The Shaved Leaves were prepared by freshly harvesting natural leaves and removing 70-80% of the trichomes from the leaf cuticle on both the abaxial and adaxial surfaces using an electric razor (P/N: XA525/42, Norelco Axe by Philips, Amsterdam, Netherlands). The Whole Leaves were freshly harvested natural leaves. Treatment diets were freshly prepared at the start of each experiment. All animals were maintained on a 16:8 hour light:dark photoperiod at 26 ˚C with ambient humidity.

Eggs were added to individual, 100 x 10 mm Petri dishes (P/N: 351029, Corning, Corning, NY); 617 hatched and were immediately provided one of the four dietary treatments. Natural leaf treatments were refreshed daily and artificial diet treatments were refreshed every three days. Larvae were monitored daily and the day of each molting event was recorded for every larva. Upon molting to fourth instar, larvae were transferred to individual, 150 x 20 mm

Petri dishes (P/N: 82.1184.500, Sarstedt, Rommelsdorf, Germany). Larvae continued to be provided their assigned diet until they reached the wandering stage.

Individuals were bathed with tap water and transferred to a 50 mL centrifuge tubes (P/N: C2750, MTC Bio, Metuchen, NJ) with a piece of a paper toweling (approximately 6 x 24 cm, P/N: 01804, Scott by Kimberly-Clark, Irving, TX) and topped with a modified cap that contained an 18% shade cloth insert to allow airflow. After 21 days, pupae were transferred into individual, eclosion cups that consisted of a deli cup with an aerated lid (P/N: D32CX, Anchor Packaging, Paragould, AR and P/N: FAB PPLID, Fabri-Kal, Kalamazoo, MI) lined with a sheet of stiff plastic climbing mesh (P/N: VX620, Frost King by Thermwell, Mahwah, NJ).

On the day of eclosion, individual moths were sexed and assigned a mate. Six mating combinations were established: 1) both parents reared on Ordinary Diet, 2) both parents reared on Trichome Diet, 3) both parents reared on Shaved Leaves, 4) both parents reared on Whole Leaves, 5) mother reared on Ordinary Diet, father reared on Whole Leaves, and 6) mother reared on Whole Leaves, father reared on Ordinary Diet. The first four mating combinations with both parents reared on the same diet were used to examine the effect of parental diet content on offspring success. The two mixed-mating combinations, along with the two, corresponding same-mating combinations were used to examine the differences inheritance of maternal and paternal dietary effects. Each mating pairs was transferred to a small mating tent containing artificial nectar (P/N: 59144, The Gatorade Company, Chicago, IL).

Twelve neonate larvae were collected from each mated pair (n = 36) and added to individual, 100 x 10 mm Petri dishes (P/N: 351029, Corning, Corning, NY). Six of these larvae were assigned to feed on Ordinary Diet and the remaining six larvae were assigned to feed on Whole Leaves. After forty-eight hours of feeding, larvae were photographed alongside a ruler. The color of each larva was measured using the blue score provided by ImageJ (Schneider et al. 2012). Larva length and width was also measured using ImageJ and these values were used to

calculate the approximate larval volume ($V = pi * r^2 * h$). This novel method eliminated the possibility of damaging the larvae due to handling and weighing to determine their size.

Offspring larvae were reared adulthood using the same method as the parental generation. Offspring mass was measured at fourth instar, fifth instar, wandering, and eclosion using an electronic pan balance and recorded to the nearest milligram. After molting to fourth and fifth instars, larvae were transferred to individual, 100 x 10 mm Petri dishes (P/N: 351029, Corning, Corning, NY) and provided one of the four treatment diets *ad libitum* for 48 hrs. Initial larva mass and the mass of diet provided were measured to the nearest 0.001 g using an electronic pan balance. After 48 hours of feeding on treatment diet, the mass of the larva, remaining diet, and all frass in the petri dish was measured to the nearest 0.001 g. These values allowed me to estimate larva growth during this period, the amount of diet they consumed, and the relative amount of frass they produced (amount of frass divided by the amount of diet consumed). I also calculated estimates of efficient conversion of diet, and metabolic gap. Conversion efficiency is the amount of ingested food converted to body weight (amount of larval weight gain divided by the amount of diet consumed). Metabolic gap is the proportion of diet consumed that is not accounted for by the amount of larval weight gain and the amount of frass produced (amount of diet consumed minus the amount of larval weight gain and the amount of frass produced; divided by the amount of diet consumed). Wet masses, as opposed to dry masses, were necessary to use because larvae could not be destructively sampled in order to monitor their growth through adulthood and reproduction. Together, the metrics for frass, conversion efficiency, and metabolic gap account for the dispensation of all diet ingested by a larva.

Larvae remained in the 150 x 20 mm Petri dishes and continued be provided their assigned treatment diets until they ceased eating at the wandering stage. Following metamorphosis, offspring survival, sex, and date of eclosion were recorded. These values were

used to determine the number of days to develop from hatching to adulthood, overall survival, and proportion of surviving females.

Statistical Analysis

Statistical analysis of the effects of parental diet on offspring traits was performed in R (R Core Team 2014) using the '*lm*' function of the base package and the post hoc comparisons were performed '*HSD.test*' function from the agricolae package (de Mendiburu 2017). Two sets of models were run: one set to examine the effect of the parental diet on offspring traits and a second set to distinguish contributions of the mother versus the father. The first set of models included three factors and their interactions: 1) Trichome Presence in parental diet (with or without trichomes), 2) Parental Diet Type (artificial diet vs. natural leaves), and 3) Offspring Diet Type (Ordinary Diet vs. Whole Leaves) to determine the effect of the contents of the parental and offspring diets. Only offspring for which both parents had been reared on the same diet were included. The second set of models included three factors and their interactions: 1) Maternal, 2) Paternal, and 3) Offspring diet types (Ordinary Diet vs. Whole Leaves) to determine the effects of diet of the mother, the father, and the offspring. Only offspring from the following mating pair combinations were examined: 1) both parents reared on Ordinary Diet, 2) mother reared on Ordinary Diet, father reared on Whole Leaves, 3) mother reared on Whole Leaves, father reared on Ordinary Diet, and 4) both parents reared on Whole Leaves.

Each model set evaluated the effects of parental diet on offspring long-term growth using separate linear regression models for effects on first instar color, first instar volume, fourth instar mass, fifth instar mass, wanderer mass, and moth mass. The effects of parental diet on offspring diet consumption and efficiency were evaluated using separate linear regression models for amount of diet consumed, larval mass gain, frass production, conversion efficiency, and metabolic gap. Each model included initial larval mass as a covariate, but omitting mass did not

qualitatively change the results. The effects of parental diet on larval development time, survival, and the proportion of females in a clutch were evaluated using separate linear regression models. Moth mass was included as a covariate in the model for development time, but omitting mass did not qualitatively change the results. Apart from color, no other factor showed significant interactions, so interaction terms were removed from all other models to conserve power. Cohen's f^2 test for effect size was calculated to compare the effect of trichome presence and of the base diet. Cohen's recommended values were used to qualify effect size (Small effect: $f^2 \geq 0.02$, Moderate effect: $f^2 \geq 0.15$, Large effect: $f^2 \geq 0.35$) (Cohen 1988).

Results

Offspring Color & Size

Color

The presence of trichomes in the parental diet did not affect the color of offspring at first instar (Parental Diet Trichome Presence (with or without trichomes): $t_{8,108} = -1.83$, $p = 0.071$; $f^2 = 0.030$). This was true regardless of the base diet on which the parents or offspring were raised (Parental Diet Type (artificial base vs natural base) x Parental Diet Trichome Presence: t8,108 = -0.60, $p = 0.553$; $f^2 = 0.045$; Offspring Diet (Ordinary Diet vs Whole Leaves) x Parental Diet Trichome Presence: $t_{8,108} = 1.21$, $p = 0.230$; Parental Diet Trichome Presence x Parental Diet Type x Offspring Diet: t8,108 = 0.76, p =0.447; $f^2 = 0.45$; Fig. 4.1). Offspring Diet significantly affected first instar larvae color and larvae reared on Whole Leaves were 50% darker than offspring reared on Ordinary Diet (Offspring Diet: $t_{8,108} = -9.86$, $p < 0.001$; $f^2 = 0.219$; Fig. 4.1). While not statistically significant at the $p < 0.05$ level, there was a small effect size, and therefore, some indication of an interaction between Parental Diet Type and Offspring Diet (Offspring Diet

x Parental Diet Type: $t_{8,108} = 1.89$, $p = 0.061$; Parental Diet Type: $t_{8,108} = -1.68$, $p = 0.097$; $f^2 = 0.021$). Data were then explored for effects of Parental diet within offspring from the two Offspring Diet treatments, separately. When offspring were reared on Whole Leaves their color differed significantly based on the diet of their parents; offspring of parents reared on natural-based diets were 8% darker in color than offspring with parents reared on artificial-based diets (Whole Leaf-fed offspring, Parental Diet Type: $t_{2,45} = 2.14$, $p = 0.038$; $f^2 = 0.102$; Fig. 4.1). However, when offspring were reared on Ordinary Diet their color did not significantly differ based on the diet of their parents (Ordinary Diet-fed offspring, Parental Diet Type: $t_{2,66} = -1.94$, $p = 0.057$; $f^2 = 0.057$). Offspring volume had no effect on offspring color (Volume: $t_{8,108} = 0.08$, $p = 0.933$).

Maternal Diet (Ordinary Diet vs Whole Leaves) did not affect the color of offspring at first instar (Maternal Diet: $t_{8,202} = -1.52$, $p = 0.130$; $f^2 = 0.029$) and there was no interaction between the Maternal Diet and the Paternal or Offspring Diets, (Maternal Diet x Paternal Diet (Ordinary Diet vs Whole Leaves): $t_{8,202} = -0.27$, $p = 0.786$; $f^2 = 0.068$; Offspring Diet (Ordinary Diet vs Whole Leaves) x Maternal Diet: $t_{8,202} = 1.08$, $p = 0.280$; Maternal Diet x Paternal Diet x Offspring Diet: $t_{8,202} = -0.04$, $p = 0.965$; $f^2 = 1.436$; Fig. 4.1).

While not statistically significant at the $p < 0.05$ level, there was a small effect, indicating a potential interaction between Paternal Diet Type and Offspring Diet (Offspring Diet x Paternal Diet: $t_{8,202} = 1.95$, $p = 0.053$; Paternal Diet: $t_{8,202} = -0.69$, $p = 0.489$; $f^2 = 0.042$). Offspring from each Offspring Diet treatment (Ordinary Diet vs Whole leaves) were tested separately for an effect of paternal Diet, revealing that offspring of fathers reared on Whole Leaves were 4% darker in color than offspring with fathers reared on Ordinary Diet, but only when offspring were reared on Whole Leaves, not when offspring were reared on Ordinary Diet (Ordinary Diet-fed offspring, Diet Type: $t_{8,103} = -0.79$, $p = 0.430$; $f^2 = 0.006$; Whole Leaf-fed offspring, Diet Type: $t_{8,102} = 2.92$, $p = 0.004$; f2 = 0.083). Overall, offspring reared on Whole Leaves were 49% darker

than offspring reared on Ordinary Diet (Offspring Diet: $t_{8,202} = -11.24$, $p < 0.001$; $f^2 = 1.402$; Fig. 4.1). Larger offspring were darker than smaller offspring (Volume: $t_{8,202} = 4.60$, $p < 0.001$).

1st Instar Volume

The volume of first instar offspring was not affected by the presence of trichomes in the parental diet or the base diet type consumed by parents or an interaction between these factors (Parental Diet Trichome Presence: $t_{4,192} = -1.69$, $p = 0.093$; $f^2 = 0.036$; Parental Diet Type: $t_{4,192} = -0.53$, $p = 0.598$; $f^2 = 0.005$; Parental Diet Type x Parental Diet Trichome Presence: $t_{4,192} = -0.23$, $p = 0.817$; $f^2 = 0.040$; Fig. 4.1). At first instar, offspring reared on Whole Leaves were 49% smaller than offspring reared on Ordinary Diet (Offspring Diet: $t_{4,192} = -10.99$, $p < 0.001$; $f^2 = 0.629$; Fig. 4.1). There was no interaction between Parental treatment and Offspring treatment (Offspring Diet x Parental Diet Trichome Presence: $t_{4,192} = 1.04$, $p = 0.302$; $f^2 = 0.006$; Offspring Diet x Parental Diet Type: $t_{4,192} = 0.12$, $p = 0.906$; $f^2 < 0.001$; Offspring Diet x Parental Diet Trichome Presence x Parental Diet Type: $t_{4,192} = 0.17$, $p = 0.865$; $f^2 < 0.001$).

The volume of first instar offspring was not significantly affected by either maternal or paternal diet or an interaction between these diets (Maternal Diet: $t_{4,206} = -0.19$, $p = 0.854$; $f^2 = 0.024$; Paternal Diet: $t_{4,206} = -0.29$, $p = 0.771$; $f^2 = 0.027$; Maternal Diet x Paternal Diet: $t_{4,206} = -10.93$, $p = 0.146$; $f^2 = 0.038$; Fig. 4.1). Again, at first instar, offspring reared on Whole Leaves were 47% smaller than offspring reared on Ordinary Diet (Offspring Diet: $t_{4,206} = -1.46$, $p < 0.001$ $f^2 = 0.580$; Fig. 4.1). There was no interaction between Parental treatments and Offspring treatment (Offspring Diet x Maternal Diet: $t_{4,206} = 0.13$, $p = 0.895$; $f^2 < 0.001$; Offspring Diet x Paternal Diet: $t_{4,206} = 1.24$, $p = 0.217$; $f^2 < 0.001$; Offspring Diet x Maternal Diet x Paternal Diet: $t_{4,206} = 0.02$, $p = 0.984$; f2 < 0.001).

4th Instar Mass

Trichome presence in the parental diet led to less massive offspring at fourth instar (17% decrease), regardless of the base diet type consumed by parents (Parental Diet Trichome Presence: $t_{4,170} = -2.72$, $p = 0.007$; $f^2 = 0.051$; Parental Diet Type x Trichome Presence: $t_{4,170} = 1.07$, $p = 0.285$; $f^2 = 0.074$; Fig. 4.1). Offspring mass at fourth instar was not affected by the base diet type consumed by parents or the diet type consumed by offspring (Parental Diet Type: $t_{4,170} = 0.57$, $p = 0.568$; $f^2 = 0.028$; Offspring Diet: $t_{4,170} = -0.46$, $p = 0.650$; $f^2 = 0.001$; Fig. 4.1). There was no interaction between Parental treatment and Offspring treatment (Offspring Diet x Parental Diet Trichome Presence: $t_{4,170} = -1.60$, $p = 0.112$; $f^2 = 0.015$; Offspring Diet x Parental Diet Type: $t_{4,170} = -0.14$, $p = 0.888$; $f^2 < 0.001$; Offspring Diet x Parental Diet Trichome Presence x Parental Diet Type: $t_{4,170} = 1.55$, $p = 0.124$; $f^2 = 0.014$).

Maternal diet did not affect offspring mass at fourth instar (Maternal Diet: $t_{4,205} = -0.69$, $p = 0.489$; $f^2 = 0.011$). Offspring of fathers reared on Whole Leaves were 8% smaller than offspring of fathers reared on Ordinary Diet, regardless of the maternal diet (Paternal Diet: $t_{4,205} = -2.03$, $p = 0.043$; $f^2 = 0.020$; Maternal Diet x Paternal Diet: $t_{4,205} = 1.47$, $p = 0.142$; $f^2 = 0.022$; Fig. 4.1). Offspring mass at fourth instar was not affected by the diet type consumed by offspring (Offspring Diet: $t_{4,205} = 0.40$, $p = 0.688$; $f^2 = 0.001$; Fig. 4.1). There was no interaction between Parental treatments and Offspring treatment (Offspring Diet x Maternal Diet: $t_{4,205} = -0.18$, $p = 0.855$; $f^2 < 0.001$; Offspring Diet x Paternal Diet: $t_{4,205} = -0.45$, $p = 0.656$; $f^2 < 0.001$; Offspring Diet x Maternal Diet x Paternal Diet: $t_{4,205} = 0.82$, $p = 0.413$; $f^2 = 0.003$).

5th Instar Mass

Trichomes present in the parental diet lead to offspring of lower mass at fourth instar (12% decrease), regardless of the base diet type consumed by parents (Parental Diet Trichome Presence: $t_{4,162} = -2.43$, $p = 0.016$; $f^2 = 0.039$; Parental Diet Type x Parental Diet Trichome Presence: $t_{4,162} = 1.24$, $p = 0.218$; $f^2 = 0.064$; Parental Diet Type: $t_{4,162} = 0.47$, $p = 0.642$; $f^2 =$

0.032; Fig. 4.1). Offspring reared on Whole Leaves were 12% smaller than offspring reared on Ordinary Diet (Offspring Diet: $t_{4,162} = -2.18$, $p = 0.031$; $f^2 = 0.029$; Fig. 4.1). There was no interaction between Parental treatment and Offspring treatment (Offspring Diet x Parental Diet Trichome Presence: $t_{4,162} = -0.71$, $p = 0.479$; $f^2 = 0.003$; Offspring Diet x Parental Diet Type: $t_{4,162} = 1.23$, $p = 0.220$; $f^2 = 0.010$; Offspring Diet x Parental Diet Trichome Presence x Parental Diet Type: $t_{4,162} = 0.51$, $p = 0.614$; $f^2 = 0.002$).

The mass of fifth instar offspring was not significantly affected by either maternal or paternal diet or an interaction between these diets (Maternal Diet: $t_{4,196} = 0.09$, $p = 0.929$; $f^2 = 0.001$; Paternal Diet: $t_{4,196} = -0.56$, $p = 0.580$; $f^2 = 0.002$; Maternal Diet x Paternal Diet: $t_{4,196} = 0.22$, $p = 0.825$; $f^2 = 0.003$; Fig. 4.1). At fifth instar, offspring reared on Whole Leaves were 12% smaller than offspring reared on Ordinary Diet (Offspring Diet: $t_{4,196} = -2.42$, $p = 0.016$; $f^2 = 0.030$; Fig. 4.1). There was no interaction between Parental treatments and Offspring treatment (Offspring Diet x Maternal Diet: $t_{4,196} = 0.48$, $p = 0.632$; $f^2 < 0.001$; Offspring Diet x Paternal Diet: $t_{4,196} = -0.35$, $p = 0.728$; $f^2 < 0.001$; Offspring Diet x Maternal Diet x Paternal Diet: $t_{4,196} = 0.79$, $p = 0.432$; $f^2 = 0.003$).

Wanderer Mass

The mass of wandering offspring was not affected by the presence of trichomes in the parental diet or the base diet type consumed by parents or interaction between these factors (Parental Diet Trichome Presence: $t_{4,154} = -1.11$, $p = 0.271$; $f^2 = 0.024$; Parental Diet Type: $t_{4,154} = 0.24$, $p = 0.812$; $f^2 = 0.001$; Parental Diet Type x Parental Diet Trichome Presence: $t_{4,154} = -0.34$, $p = 0.734$; $f^2 = 0.024$; Fig. 4.1). At wandering, offspring reared on Whole Leaves were 19% smaller than offspring reared on Ordinary Diet (Offspring Diet: $t_{4,154} = -9.39$, $p < 0.001$; $f^2 = 0.572$; Fig. 4.1). There was no interaction between Parental treatment and Offspring treatment (Offspring

Diet x Parental Diet Trichome Presence: $t_{4,154} = -1.41$, $p = 0.162$; $f^2 = 0.013$; Offspring Diet x Parental Diet Type: $t_{4,154} = -0.49$, $p = 0.623$; $f^2 = 0.002$; Offspring Diet x Parental Diet Trichome Presence x Parental Diet Type: $t_{4,154} = 1.27$, $p = 0.205$ $f^2 = 0.011$).

The mass of wandering offspring was not significantly affected by either maternal or paternal diet or an interaction between these diets (Maternal Diet: $t_{4,189} = -0.76$, $p = 0.446$; $f^2 = 0.015$; Paternal Diet: $t_{4,189} = 0.24$, $p = 0.810$; $f^2 = 0.003$; Maternal Diet x Paternal Diet: $t_{4,189} = -0.61$, $p = 0.540$; $f^2 = 0.015$; Fig. 4.1). Again, at wandering, offspring reared on Whole Leaves were 15% smaller than offspring reared on Ordinary Diet (Offspring Diet (Ordinary vs Whole): $t_{4,189} = -7.65$, $p < 0.001$; $f^2 = 0.309$; Fig. 4.1). There was no interaction between Parental treatments and Offspring treatment (Offspring Diet x Maternal Diet: $t_{4,189} = 0.93$, $p = 0.352$; $f^2 < 0.001$; Offspring Diet x Paternal Diet: $t_{4,189} = -0.77$, $p = 0.441$; $f^2 < 0.001$; Offspring Diet x Maternal Diet x Paternal Diet: $t_{4,189} = -0.12$, $p = 0.903$; $f^2 < 0.001$).

Moth Mass

The mass of adult moth offspring was not affected by the presence of trichomes in the parental diet or the base diet type consumed by parents or interaction between these factors (Parental Diet Trichome Presence: $t_{4,130} = -1.05$, $p = 0.297$; $f^2 = 0.009$; Parental Diet Type: $t_{4,130} = -0.15$, $p = 0.880$; $f^2 = 0.003$; Parental Diet Type x Parental Diet Trichome Presence: $t_{4,130} = 0.58$, $p = 0.566$; $f^2 = 0.010$; Fig. 4.1). In adulthood, offspring reared on Whole Leaves were 37% smaller than offspring reared on Ordinary Diet (Offspring Diet: $t_{4,130} = -10.48$, $p < 0.001$; $f^2 = 0.844$; Fig. 4.1). There was no interaction between Parental treatment and Offspring treatment (Offspring Diet x Parental Diet Trichome Presence: $t_{4,130} = -0.34$, $p = 0.739$; $f^2 < 0.001$; Offspring Diet x Parental Diet Type: $t_{4,130} = -0.31$, $p = 0.754$; $f^2 < 0.001$; Offspring Diet x Parental Diet Trichome Presence x Parental Diet Type: $t_{4,130} = 0.94$, $p = 0.347$; $f^2 = 0.007$).

The mass of adult moth offspring was not significantly affected by either maternal or paternal diet or an interaction between these diets (Maternal Diet: $t_{4,156} = -1.63$, $p = 0.104$; $f^2 = 0.017$; Paternal Diet: $t_{4,156} = -0.58$, $p = 0.566$; $f^2 = 0.011$; Maternal Diet x Paternal Diet: $t_{4,156} = 1.22$, $p = 0.225$; $f^2 = 0.020$; Fig. 4.1). Again, in adulthood offspring reared on Whole Leaves were 32% smaller than offspring reared on Ordinary Diet (Offspring Diet (Ordinary vs Whole): $t_{4,156} = -9.99$, $p < 0.001$; $f^2 = 0.640$; Fig. 4.1). There was no interaction between Parental treatments and Offspring treatment (Offspring Diet x Maternal Diet: $t_{4,156} = 1.91$, $p = 0.058$; $f^2 < 0.001$; Offspring Diet x Paternal Diet: $t_{4,156} = -0.06$, $p = 0.950$; $f^2 < 0.001$; Offspring Diet x Maternal Diet x Paternal Diet: $t_{4,156} = -0.66$, $p = 0.512$; $f^2 = 0.003$).

Offspring Diet Consumption & Efficiency

Diet Consumed

Offspring consumed more diet if their parents consumed trichomes (6% increase), but only if the trichomes were on a natural-based diet (whole leaves) (Parental Diet Type x Parental Diet Trichome Presence: $t_{5,335} = 2.55$, $p = 0.011$; $f^2 = 0.025$; Parental Diet Trichome Presence: $t_{5,335} = -1.06$, $p = 0.292$; $f^2 = 0.023$; Fig. 3.2). Offspring diet consumption was not affected by the base diet type consumed by parents (Parental Diet Type: $t_{5,335} = -1.19$, $p = 0.234$; $f^2 = 0.022$; Fig. 3.2). Offspring reared on Whole Leaves consumed 20% more diet than offspring reared on Ordinary Diet (Offspring Diet (Ordinary vs Whole): $t_{5,335} = 9.31$, $p = < 0.001$; $f^2 = 0.259$; Fig. 3.2). There was no interaction between Parental treatment and Offspring treatment (Offspring Diet x Parental Diet Trichome Presence: $t_{5,335} = -0.43$, $p = 0.666$; $f^2 < 0.001$; Offspring Diet x Parental Diet Type: $t_{5,335} = 1.20$, $p = 0.232$; $f^2 = 0.004$; Offspring Diet x Parental Diet Trichome Presence x Parental Diet Type: $t_{5,335} = 0.97$, $p = 0.335$; $f^2 = 0.003$). Larger offspring consumed more diet than smaller offspring (Weight: $t_{5,335} = 45.76$, $p < 0.001$).

The amount of diet consumed by offspring depended upon the interaction between the effects of their mother's and father's diets (Maternal Diet x Paternal Diet: $t_{5,404} = 2.12$, $p = 0.035$; $f^2 = 0.017$; Maternal Diet (Ordinary vs Whole): $t_{5,404} = -0.34$, $p = 0.737$; $f^2 = 0.016$; Paternal Diet (Ordinary vs Whole): $t_{5,404} = -1.24$, $p = 0.218$; $f^2 = 0.012$; Fig. 3.2). Offspring of fathers reared on Whole Leaves consumed 15% more diet if their mothers were reared on Whole Leaves as compared to offspring with fathers reared on Whole Leaves and mothers reared on Ordinary Diet. Offspring of fathers reared on Ordinary Diet consumed the same amount, regardless of their mother's diet. Offspring reared on Whole Leaves consumed 20% more diet than offspring reared on Ordinary Diet (Offspring Diet (Ordinary vs Whole): $t_{5,404} = 10.04$, $p < 0.001$; $f^2 = 0.250$; Fig. 3.2). There was no interaction between Parental treatments and Offspring treatment (Offspring Diet x Maternal Diet: $t_{5,404} = 0.94$, $p = 0.347$; $f^2 < 0.001$; Offspring Diet x Paternal Diet: $t_{5,404} = -0.45$, $p = 0.654$; $f^2 < 0.001$; Offspring Diet x Maternal Diet x Paternal Diet: $t_{5,404} = 0.76$, $p = 0.446$; $f^2 = 0.001$). Larger offspring consumed more diet than smaller offspring (Weight: $t_{5,404} = 49.55$, $p < 0.001$).

Larval Mass Gained

The mass gained by offspring was not affected by the presence of trichomes in the parental diet or the base diet type consumed by parents or an interaction between these factors (Parental Diet Trichome Presence: $t_{5,335} = -1.05$, $p = 0.296$; $f^2 = 0.003$; Diet Type: $t_{5,335} = -1.39$, $p = 0.167$; $f^2 = 0.006$; Parental Diet Type x Parental Diet Trichome Presence: $t_{5,335} = 0.65$, $p = 0.515$; $f^2 = 0.008$; Fig. 3.2). Offspring reared on Whole Leaves gained 34% less mass than offspring reared on Ordinary Diet (Offspring Diet: $t_{5,335} = -9.32$, $p < 0.001$; $f^2 = 0.260$; Fig. 3.2). There was no interaction between Parental treatment and Offspring treatment (Offspring Diet x Parental Diet Trichome Presence: $t_{5,335} = 0.91$, $p = 0.364$; $f^2 = 0.002$; Offspring Diet x Paternal Diet: $t_{5,335} = 0.69$, $p = 0.492$; $f^2 = 0.001$; Offspring Diet x Parental Diet Trichome Presence x

Paternal Diet: $t_{5,335} = -0.57$, $p = 0.566$; $f^2 < 0.001$). Larger offspring gained more mass than smaller offspring (Weight: $t_{5,335} = 43.63$, $p < 0.001$).

The mass gained by offspring was not significantly affected by either maternal or paternal diet or an interaction between these diets (Maternal Diet: $t_{5,404} = -1.53$, $p = 0.126$; $f^2 = 0.006$; Paternal Diet: $t_{5,404} = -1.02$, $p = 0.307$; $f^2 = 0.003$; Maternal Diet x Paternal Diet: $t_{5,404} = 0.78$, $p = 0.439$; $f^2 = 0.007$; Fig. 3.2). Offspring reared on Whole Leaves gained 33% less mass than offspring reared on Ordinary Diet (Offspring Diet: t5,404 = -8.88, $p < 0.001$; $f^2 = 0.195$; Fig. 3.2). There was no interaction between Parental treatments and Offspring treatment (Offspring Diet x Maternal Diet: $t_{5,404} = 0.94$, $p = 0.350$; $f^2 < 0.001$; Offspring Diet x Paternal Diet: $t_{5,404} = -0.13$, $p = 0.898$; $f^2 < 0.001$; Offspring Diet x Maternal Diet x Paternal Diet: $t_{5,404} = 0.17$, $p = 0.862$; $f^2 < 0.001$). Larger offspring gained more mass than smaller offspring (Weight: $t = 42.69$, $p < 0.001$).

Diet Excreted as Frass

The diet excreted as frass by offspring was not affected by the presence of trichomes in the parental diet or the base diet type consumed by parents or interaction between these factors (Parental Diet Trichome Presence: $t_{5,335} = 1.21$, $p = 0.226$; $f^2 = 0.005$; Parental Diet Type: $t_{5,335} = 0.52$, $p = 0.602$; $f^2 = 0.005$; Parental Diet Type x Parental Diet Trichome Presence: $t_{5,335} = -1.22$, $p = 0.222$; $f^2 = 0.006$; Fig. 3.2). Offspring reared on Whole Leaves produced a 32% higher proportion of frass than offspring reared on Ordinary Diet (Offspring Diet: $t_{5,335} = 10.39$, $p < 0.001$; $f^2 = 0.322$; Fig. 3.2). There was no interaction between Parental treatment and Offspring treatment (Offspring Diet x Parental Diet Trichome Presence: $t_{5,335} = 0.76$, $p = 0.450$; f2 = 0.002; Offspring Diet x Parental Diet Type: $t_{5,335} = 0.86$, $p = 0.393$; $f^2 = 0.002$; Offspring Diet x Parental Diet Trichome Presence x Parental Diet Type: $t_{5,335} = -1.58$, $p = 0.115$; $f^2 = 0.008$). Larger

offspring produced a higher proportion of frass than smaller offspring (Weight: $t_{5,335} = 21.92$, $p < 0.001$).

The diet excreted as frass by offspring was not significantly affected by either maternal or paternal diet or an interaction between these diets (Maternal Diet: $t_{5,404} = 0.16$, $p = 0.877$; $f^2 = 0.002$; Paternal Diet: $t_{5,404} = -0.97$, $p = 0.333$; $f^2 = 0.002$; Maternal Diet x Paternal Diet: $t_{5,404} = -0.60$, $p = 0.548$; $f^2 = 0.004$; Fig. 3.2). Offspring reared on Whole Leaves produced a 26% higher proportion of frass than offspring reared on Ordinary Diet (Offspring Diet: t5,404 = 9.61, $p < 0.001$; $f^2 = 0.229$; Fig. 3.2). There was no interaction between Parental treatments and Offspring treatment (Offspring Diet x Maternal Diet: $t_{5,404} = 0.41$, $p = 0.684$; $f^2 < 0.001$; Offspring Diet x Paternal Diet: $t_{5,404} = -0.92$, $p = 0.359$; $f^2 < 0.001$; Offspring Diet x Maternal Diet x Paternal Diet: $t_{5,404} = 0.51$, $p = 0.608$; $f^2 < 0.001$). Larger offspring produced a higher proportion of frass than smaller offspring (Weight: $t_{5,404} = -25.31$, $p < 0.001$).

Conversion Efficiency

Offspring had a lower efficiency of converting diet to body mass if their parents consumed trichomes (13% decrease), but only if the trichomes were on a natural-based diet (Whole Leaves) (Parental Diet Type x Parental Diet Trichome Presence: $t_{5,335} = -2.19$, $p = 0.029$; $f^2 = 0.043$; Parental Diet Trichome Presence: $t_{5,335} = -0.65$, $p = 0.514$; $f^2 = 0.042$; Fig. 3.2). Offspring conversion efficiency was not affected by the base diet type consumed by parents (Parental Diet Type: $t_{5,335} = 1.68$, $p = 0.095$; $f^2 = 0.014$; Fig. 3.2). Offspring reared on Whole Leaves had a 46% lower conversion efficiency than offspring reared on Ordinary Diet (Offspring Diet: $t_{5,335} = -24.18$, $p < 0.001$; $f^2 = 0.118$; Fig. 3.2). There was no interaction between Parental treatment and Offspring treatment (Offspring Diet x Parental Diet Trichome Presence: $t_{5,335} = -0.03$, $p = 0.975$; $f^2 < 0.001$; Offspring Diet x Parental Diet Type: $t_{5,335} = -0.65$, $p = 0.519$; $f^2 = 0.001$; Offspring Diet x Parental Diet Trichome Presence x Parental Diet Type: $t_{5,335} = 0.79$, $p =$

0.430; $f^2 = 0.002$). Larger offspring had a higher conversion efficiency than smaller offspring (Weight: $t_{5,335} = 6.29$, $p < 0.001$).

The conversion efficiency of offspring was not significantly affected by either maternal or paternal diet or an interaction between these diets (Maternal Diet: $t_{5,404} = -0.32$, $p = 0.750$; $f^2 = 0.002$; Paternal Diet: $t_{5,404} = -1.21$, $p = 0.229$; $f^2 = 0.013$; Maternal Diet x Paternal Diet: $t_{5,404} = -0.41$, $p = 0.680$; $f^2 = 0.013$; Fig. 3.2). Offspring reared on Whole Leaves had a 46% lower conversion efficiency than offspring reared on Ordinary Diet (Offspring Diet: $t_{5,404} = -25.03$, $p < 0.001$; $f^2 = 1.550$; Fig. 3.2). There was no interaction between Parental treatments and Offspring treatment (Offspring Diet x Maternal Diet: $t_{5,404} = 0.19$, $p = 0.848$; $f^2 < 0.001$; Offspring Diet x Paternal Diet: $t_{5,404} = 0.47$, $p = 0.639$; $f^2 < 0.001$; Offspring Diet x Maternal Diet x Paternal Diet: $t_{5,404} = 0.01$, $p = 0.993$; $f^2 < 0.001$). Larger offspring had a higher conversion efficiency than smaller offspring (Weight: $t_{5,404} = 5.64$, $p < 0.001$).

Metabolic Gap

Offspring had a greater metabolic gap if their parents consumed trichomes (18% increase), but only if the trichomes were on a natural-based diet (whole leaves) (Parental Diet Type x Parental Diet Trichome Presence: $t_{5,335} = 2.02$, $p = 0.044$; $f^2 = 0.017$; Parental Diet Trichome Presence: $t_{5,335} = -0.50$, $p = 0.515$; $f^2 = 0.017$; Fig. 3.2). Offspring metabolic gap was not affected by the base diet type consumed by parents (Parental Diet Type: $t_{5,335} = -1.26$, $p = 0.210$; $f^2 = 0.012$; Fig. 3.2). Offspring reared on Whole Leaves had a 31% greater metabolic gap than offspring reared on Ordinary Diet (Offspring Diet: $t_{5,335} = 5.55$, $p < 0.001$; $f^2 = 0.092$; Fig. 3.2). There was no interaction between Parental treatment and Offspring treatment (Offspring Diet x Parental Diet Trichome Presence: $t_{5,335} = -0.51$, $p = 0.610$; $f^2 < 0.001$; Offspring Diet x Parental Diet Type: $t_{5,335} = -0.26$, $p = 0.799$; $f^2 < 0.001$; Offspring Diet x Parental Diet Trichome

Presence x Parental Diet Type: $t_{5,335} = 0.68$, $p = 0.494$; $f^2 = 0.001$). Larger offspring had a smaller metabolic gap than smaller offspring (Weight: $t_{5,335} = -18.69$, $p < 0.001$).

The metabolic gap of offspring was not significantly affected by either maternal or paternal diet or an interaction between these diets (Maternal Diet: $t_{5,404} = 0.06$, $p = 0.949$; $f^2 = 0.001$; Paternal Diet: $t_{5,404} = 1.29$, $p = 0.199$; $f^2 = 0.007$; Maternal Diet x Paternal Diet: $t_{5,404} = -0.19$, $p = 0.852$; $f^2 = 0.008$; Fig. 3.2). Offspring reared on Whole Leaves had a 34% greater metabolic gap than offspring reared on Ordinary Diet (Offspring Diet: $t_{5,404} = 6.73$, $p < 0.001$; $f^2 = 0.112$; Fig. 3.2). Larger offspring had a smaller metabolic gap than smaller offspring (Weight: t5,404 = -19.98, $p < 0.001$). There was no interaction between Parental treatments and Offspring treatment (Offspring Diet x Maternal Diet: $t_{5,404} = -0.39$, $p = 0.700$; $f^2 < 0.001$; Offspring Diet x Paternal Diet: $t_{5,404} = 0.52$, $p = 0.603$; $f^2 < 0.001$; Offspring Diet x Maternal Diet x Paternal Diet: $t_{5,404} = -0.40$, $p = 0.692$; $f^2 < 0.001$).

Offspring Development, Survival, & Reproduction

Development to adulthood

The development time of offspring (number of days from hatching to eclosion) was not affected by the presence of trichomes in the parental diet or the base diet type consumed by parents or interaction between these factors (Parental Diet Trichome Presence: $t_{5,117} = -1.29$, $p = 0.201$; $f^2 = 0.024$; Parental Diet Type: $t_{5,117} = -0.89$, $p = 0.377$; $f^2 = 0.010$; Parental Diet Type x Parental Diet Trichome Presence: $t_{5,117} = 0.16$, $p = 0.875$; $f^2 = 0.032$; Fig. 4.3). Offspring reared on Whole Leaves required 1.7 more days (3% increase) to reach adulthood than offspring reared on Ordinary Diet (Offspring Diet: $t_{5,117} = 4.87$, $p < 0.001$; $f^2 = 0.202$; Fig. 4.3). There was no interaction between Parental treatment and Offspring treatment (Offspring Diet x Parental Diet Trichome Presence: $t_{5,117} = -0.13$, $p = 0.896$; $f^2 < 0.001$; Offspring Diet x Parental Diet Type: $t_{5,117}$

= -1.10, p = 0.275; f^2 = 0.011; Offspring Diet x Parental Diet Trichome Presence x Parental Diet Type: $t_{5,117}$ = 0.68, p = 0.495; f^2 = 0.004). Larger offspring required more days to reach adulthood than smaller offspring (Weight: $t_{5,117}$ = 3.32, p = 0.001).

The development time of offspring was not significantly affected by either maternal or paternal diet or an interaction between these diets (Maternal Diet: $t_{5,143}$ = -1.58, p = 0.117; f^2 = 0.042; Paternal Diet: $t_{5,143}$ = -0.09, p = 0.926; f^2 = 0.002; Maternal Diet x Paternal Diet: $t_{5,143}$ = -0.31, p = 0.758; f^2 = 0.042; Fig. 4.3). Offspring reared on Whole Leaves required 2.33 more days (5% increase) to reach adulthood than offspring reared on Ordinary Diet (Offspring Diet: $t_{5,143}$ = 6.16, p < 0.001; f^2 = 0.265; Fig. 4.3). There was no interaction between Parental treatments and Offspring treatment (Offspring Diet x Maternal Diet: $t_{5,143}$ = 0.16, p = 0.875; f^2 < 0.001; Offspring Diet x Paternal Diet: $t_{5,143}$ = 0.01, p = 0.989; f^2 < 0.001; Offspring Diet x Maternal Diet x Paternal Diet: $t_{5,143}$ = -0.27, p = 0.790; f^2 < 0.001). Larger offspring required more days to reach adulthood than smaller offspring (Weight: $t_{5,143}$ = 2.62, p = 0.010).

Survival to adulthood

The presence of trichomes in the parental diet increased offspring survival by 76%, when the trichomes were consumed in artificial diet. However, when parents consumed trichomes *in situ* on leaves, offspring survival was decreased by 16% (Parental Diet Type x Parental Diet Trichome Presence: $t_{4,258}$ = -2.85, p = 0.005; f^2 = 0.047; Parental Diet Trichome Presence: $t_{4,258}$ = 3.08, p = 0.002; f^2 = 0.041; Fig. 4.3). Offspring of parents reared on natural-based diet were 17% more likely to survive than offspring of parents reared on artificial-based diet (Parental Diet Type: $t_{4,258}$ = 2.74, p = 0.006; f^2 = 0.036; Fig. 4.3). Offspring reared on Whole Leaves were 36% less likely to survive to adulthood than offspring reared on Ordinary Diet (Offspring Diet: $t_{4,258}$ = -3.51, p = 0.001; f^2 = 0.047; Fig. 4.3). There was no interaction between Parental treatment and Offspring treatment (Offspring Diet x Parental Diet Trichome Presence: $t_{4,258}$ = -0.68, p = 0.499;

$f^2 = 0.002$; Offspring Diet x Parental Diet Type: $t_{4,258} = -1.32$, $p = 0.188$; $f^2 = 0.007$; Offspring Diet x Parental Diet Trichome Presence x Parental Diet Type: $t_{4,258} = 0.64$, $p = 0.521$; $f^2 = 0.002$).

Offspring of mothers reared on Whole Leaves were 51% more likely to survive to adulthood, and while not significant, there was a slight trend for offspring of fathers reared on Whole Leaves to be more likely to survive to adulthood (Maternal Diet: $t_{4,301} = 4.84$, $p = < 0.001$; $f^2 = 0.078$; Paternal Diet: $t_{4,301} = 1.71$, $p = 0.089$; $f^2 = 0.035$; Fig. 4.3). However, when both parents were reared on Whole Leaves, offspring were significantly less likely to survive to adulthood (Maternal Diet x Paternal Diet: $t_{4,301} = -3.19$, $p = 0.002$; $f^2 = 0.081$; Fig. 4.3). Offspring reared on Whole Leaves were less likely to survive to adulthood than offspring reared on Ordinary Diet (Offspring Diet: $t_{4,301} = -3.11$, $p = 0.002$; $f^2 = 0.081$; Fig. 4.3). There was no interaction between Parental treatments and Offspring treatment (Offspring Diet x Maternal Diet: $t_{4,301} = -0.97$, $p = 0.334$; $f^2 < 0.001$; Offspring Diet x Paternal Diet: $t_{4,301} = -0.05$, $p = 0.961$; $f^2 < 0.001$; Offspring Diet x Maternal Diet x Paternal Diet: $t_{4,301} = -0.10$, $p = 0.924$; $f^2 < 0.001$).

Proportion of females from initial population

The proportion of females that survived to adulthood in each offspring clutch was increased when trichomes were present in the parental diet (93% increase), regardless of the base diet type consumed by the parents (Parental Diet Trichome Presence: $t_{4,35} = 2.21$, $p = 0.034$; $f^2 = 0.143$; Parental Diet Type: $t_{4,35} = 1.96$, $p = 0.058$; $f^2 = 0.111$; Parental Diet Type x Parental Diet Trichome Presence: $t_{4,35} = -1.24$, $p = 0.223$; $f^2 = 0.249$; Fig. 4.3). Female offspring were 52% less likely to survive when reared on Whole Leaves when reared on Ordinary Diet (Offspring Diet: $t_{4,35} = -2.34$, $p = 0.025$; $f^2 = 0.156$; Fig. 4.3). There was no interaction between Parental treatment and Offspring treatment (Offspring Diet x Parental Diet Trichome Presence: $t_{4,35} = -0.31$, $p = 0.761$; $f^2 = 0.003$; Offspring Diet x Parental Diet Type: $t_{4,35} = -0.55$, $p = 0.590$; $f^2 = 0.009$;

Offspring Diet x Parental Diet Trichome Presence x Parental Diet Type: $t_{4,35} = -0.05$, $p = 0.960$; $f^2 < 0.001$).

Female offspring of mothers reared on Whole Leaves were more 175% likely to survive than female offspring of mothers reared on Ordinary Diet, regardless of the paternal diet (Maternal Diet: $t_{4,45} = 2.86$, $p = 0.006$; $f^2 = 0.239$; Maternal Diet x Paternal Diet: $t_{4,45} = -0.68$, $p = 0.498$; $f^2 = 0.240$; Fig. 4.3). Paternal Diet and Offspring Diet had no effect on the proportion of females surviving to adulthood (Paternal Diet: $t_{4,45} = 0.68$, $p = 0.503$; $f^2 = 0.012$; Offspring Diet: $t_{4,45} = -1.13$, $p = 0.266$; $f^2 = 0.028$; Fig. 4.3). There was no interaction between Parental treatments and Offspring treatment (Offspring Diet x Maternal Diet: $t_{4,45} = 0.75$, $p = 0.458$; $f^2 < 0.001$; Offspring Diet x Paternal Diet: $t_{4,45} = 0.10$, $p = 0.924$; $f^2 < 0.001$; Offspring Diet x Maternal Diet x Paternal Diet: $t_{4,45} = -1.24$, $p = 0.223$; $f^2 = 0.036$).

Discussion

The effects of consuming horsenettle trichomes spanned generations of *Manduca sexta*. Trichomes in the diet consumed by the parental generation led to differences in offspring size, metabolism, and survival. The base diet type consumed by the parents, artificial diet or natural leaves, led to differences in offspring color, metabolism, and survival. The individual maternal and paternal dietary contributions to offspring were slightly less distinguishable. Maternal diet affected offspring diet consumption and survival. Paternal diet affected offspring color, mass, diet consumption, and survival. Offspring diet had no interaction with parental diet, but independently affected offspring color, size, metabolism, development time, and survival. The effects of parental diet and the contributions from each parent were monitored in offspring in three categories: color and size, diet consumption and efficiency, and development and survival.

Trichomes consumed by parents did not lead to transgenerational effects on offspring color, however offspring of parents reared on natural-based diets were darker than offspring of parents reared on artificial diets. This color difference is likely to have been passed from the father. Furthermore, offspring diet also affected offspring color; offspring reared on Whole Leaves were darker than offspring reared on Ordinary Diet. Color differences among offspring can be passed from parents through genetics or through the provisioning of xanthophyll sequestered from the parental diet (Dahlman 1969, Kawooya et al. 1985). In this study, the difference in offspring color is likely caused by dietary xanthophyll acquired by the parents and passed to offspring, because offspring color varied between offspring of parents reared on natural and artificial diets and because offspring diet also affected offspring color.

Trichomes consumed by parents led to reduced offspring mass at fourth and fifth instars, regardless of the type of diet consumed by offspring, but the base diet type consumed by parents had no affect offspring size. Size differences in offspring due to parental diet were likely inherited from the father. While parental diet affected offspring mass throughout the larval phase, by adulthood only the offspring diet affected offspring mass. This is as expected, because while the parental diet may influence offspring through egg provisioning or epigenetic regulation of offspring metabolism, the diet consumed by the offspring provides the raw material for growth (Ojeda-Avila et al. 2003, Raguso et al. 2007).

Variation in offspring size is likely due to differences in offspring diet consumption and diet conversion efficiency. Offspring of larvae that had consumed trichomes on natural leaves ate more diet but gained the same amount of mass and produced the same amount of frass as offspring of larvae that had not consumed trichomes. They also had lower conversion efficiencies and higher metabolic gaps. Together, this indicates that nutritional energy from the additional diet consumed by the offspring was diverted from growth and utilized in other metabolic processes (Freitak et al. 2003, Taiz and Zeiger 2010, Fürstenberg-Hägg et al. 2013). Maternal and paternal

dietary effects were indistinguishable for all diet conversion efficiency metrics, except for diet consumption, in which both parental diets affected offspring consumption. Offspring diet also affected consumption and fit a similar pattern to the effects of trichomes consumed in the parental diet. Offspring reared on Whole Leaves (which contained trichomes and natural plant chemistry) ate more diet, gained less mass, and produced more frass than offspring reared on Artificial Diet. Furthermore, offspring reared on Whole Leaves had lower conversion efficiencies and higher metabolic gaps and it is also likely that energy was diverted from growth to other metabolic processes (Freitak et al. 2003, Taiz and Zeiger 2010, Fürstenberg-Hägg et al. 2013).

The increase in offspring metabolic gap caused by trichomes consumed in the parental generation or the offspring generation may have been the result of internal damage which required the expression of tissue repair and immune response genes (Pechan et al. 2002, Fescemyer et al. 2013). Such changes to gene expression could be epigenetically inherited by offspring in the next generation to prepare them to face similar challenges within their own environment (Freitak et al. 2009, Triggs and Knell 2012, Love et al. 2013, Dew-Budd et al. 2016). Offspring prepared for their environment through parental effects like epigenetic inheritances are expected to benefit from increased survival and reproduction (Mousseau and Dingle 1991, Mousseau 1998, Agrawal et al. 1999).

Survival may be enhanced through epigenetic inheritances in two ways. Firstly, offspring born with an activated gene suite that metabolically compliments their available dietary sources would be at an advantage because their increased conversion efficiency would allow them to develop more quickly (Rossiter 1991a, 1991b, Triggs and Knell 2012). Accelerated development would reduce their exposure to pathogens, predators, and parasitoids (Kingsolver et al. 2012). However, in this experiment, the conversion efficiency was actually decreased and development time did not differ between the offspring of parents that had consumed diets with or without trichomes. Therefore, it is unlikely that trichomes consumed by parents would have affected

offspring survival by an extended development time, even if this experiment had allowed natural exposure to pathogens, predators, or parasitoids.

Alternatively, offspring may inherit an activated gene suite that metabolically prepares them to face the same environmental challenges as their parents (Triggs and Knell 2012, Freitak et al. 2014, McCormick et al. 2019). In this scenario offspring could be born ready to repair tissue damage or mount an antibiotic defense appropriate for the same plant defenses their parents consumed (Freitak et al. 2009, Triggs and Knell 2012) and therefore would be less likely to succumb after consuming diets with similar plant defenses. In this experiment, offspring of parents that consumed trichomes were more likely to survive to adulthood than offspring of parents that had not consumed trichomes, regardless of the diet they themselves consumed. This is underscored by the increased metabolic gap among offspring of parents that consumed trichomes as compared to offspring of parents that had not consumed trichomes. Increases in offspring survival and metabolic gap due to parental trichome consumption occurred regardless of the offspring diet type. In contrast, offspring reared on diet containing trichomes were less likely to survive to adulthood, indicating that they were likely unprepared to handle the internal damage caused by consuming trichomes.

Finally, trichomes consumed in the parental generation led to a higher proportion of female offspring that survived to adulthood and this effect was likely inherited from the mother. Natural leaves consumed in the parental generation did not have a similar effect. Significant increases in female survival could ultimately lead to increases in the reproductive output of the population. Differences in sex survival ratios also hints at sex-specific effects of consuming trichomes in the diet which can be investigated in future studies (Rossiter 1991a, 1991b, Triggs and Knell 2012).

Overall, trichomes consumed by hornworms led to a variety of effects on their subsequent offspring, however, trichomes *in situ* on leaves more often led to significant

differences among offspring than trichomes added to artificial diets. For example, there was a significant difference in the amount of diet consumed by offspring of parents that consumed Shaved Leaves and offspring of parents that consumed Whole Leaves, but there was no significant difference in the amount of diet consumed by the offspring of parents that consumed Ordinary Diet and Trichome Diet. This may be due to the perpendicular orientation of *in situ* trichomes versus the random orientation of added trichomes relative to the alimentary canal of larvae (Levin 1973). In the case of added trichomes to artificial diet, some trichomes may enter the larvae body parallel to the alimentary canal, lessening the opportunity to damage the larval gut. In contrast, *in situ* trichomes are consumed perpendicularly to the larval mouth and may provide more opportunities to damage internal structures (Levin 1973). Alternatively, *in situ* trichomes are accompanied by a full complement of leaf defensive compounds (Fraenkel 1959, Cipollini and Levey 1997, Wittstock and Gershenzon 2002, Fürstenberg-Hägg et al. 2013). Any internal damage dealt by *in situ* trichomes could be compounded by chemical damage in a way that added trichomes could not be compounded by artificial diet (Pechan et al. 2002, Barbeta et al. 2008, Fescemyer et al. 2013).

While parental diet affected offspring, the diet directly consumed by offspring had a greater effect on offspring for nearly every metric analyzed. This aligns with other studies of transgenerational effects among insects (Rossiter 1991b, Newcombe et al. 2015, Mbande et al. 2018). The diet consumed by offspring may have a more direct effect by supplying the nutrients for offspring growth and presenting immediate challenges, like plant defenses, for offspring to overcome (Ojeda-Avila et al. 2003, Raguso et al. 2007). Studies among vertebrates tend to show the opposite trend, with parental life history having more effect on offspring phenotype than the experience of the offspring (Catalano 2003, Lumey et al. 2011, McCormick et al. 2017, Owen et al. 2018). Future work will be needed to reconcile these disparate life history strategies.

What may be more surprising is that there was never a significant interaction between the effect of parental diet and offspring diet. Theoretically, beneficial parental effects prepare offspring to face environmental conditions similar to those experienced by the parents (Roach and Wulff 1987, Mousseau and Dingle 1991, Freitak et al. 2014). In this study, however, offspring of parents that had consumed trichomes performed better than other offspring, regardless of the diet the offspring consumed and not only when facing the same dietary challenges as their parents. Other studies of diet-mediated parental effects among insects show similar patterns of harsh maternal diets leading to offspring benefits, regardless of the diet of the offspring (Fox et al. 1995, Newcombe et al. 2015).

The finding that parental diets did not prepare their offspring to consume the same diet begs the question, what universal advantages did parents that consumed trichomes pass on to their offspring? If offspring of parents that consumed trichomes did not have a particular advantage when consuming diets containing trichomes, then it is unlikely that the advantage is directly related to consuming trichomes. Instead, parents that consumed trichomes likely conferred a general advantage that benefits offspring regardless of the diet they themselves consumed. Possible advantages could stem from genetic, phenotypic, epigenetic, or provisional mechanisms (Roach and Wulff 1987, Mousseau and Dingle 1991, Freitak et al. 2014). This study did not examine genetic differences, which are unlikely to be affected by diet, and it controlled for phenotypic differences such as chorion strength and oviposition by utilizing the uniform environmental conditions of a laboratory. It is possible that trichomes consumed by mothers affected provisioning within the eggs. As previously noted, maternal diet affected the color of eggs, which is associated with differences in provisioning (Fox et al. 1995). Differences in egg contents or quality of nutrients may account for differences observed in female survival (Rossiter 1991a). Typically, egg provisioning is associated with maternal diet and is unlikely to explain the observations of paternal diet effects on offspring color, mass, and overall survival (Roach and

Wulff 1987, Mousseau 1998). When elements of the paternal environment, such as diet, affect offspring it is typically a sign of epigenetic inheritance or inherited gene expression modifications of nuclear DNA (Freitak et al. 2009, Triggs and Knell 2012, Cahenzli and Erhardt 2013, Love et al. 2013, Dew-Budd et al. 2016). This could also explain the increases in metabolic gap among offspring of parents that consumed trichomes. Future studies will need to determine which genes have upregulated expression and should explain why the offspring of parents that consumed trichomes had a universal advantage over their peers, regardless of the diet offspring themselves consumed.

Shifting perspectives to the plant host's point of view, in the first generation trichome consumption lead to potential benefits for the plant host. When trichomes were present in the diet, this included a reduction in the amount of leaf tissue consumed by larvae, potential reduction in the number of larvae feeding on leaves, and potentially reducing the number of viable offspring. In the second generation, those same trichomes consumed by hornworms led to effects on the offspring of those hornworms. However, now the larvae were prepared for their environment and consumed more diet and were more likely to survive and continue feeding. There was also an indication that reproduction would increase in the second generation due to the increased survival of females. Clearly there is a tradeoff in which the plants are able to defend themselves from herbivory by the first generation of hornworms, but the second generation of hornworms is able to withstand the plant defenses. As herbivory increases, plants induce greater defense production and the cycle continues throughout the season (Agrawal 1999, Thaler 1999a, 1999b, Kariyat et al. 2013).

Over the course of a single summer, the hornworms, which are multivoltine, may produce three generations of offspring, further extending the back and forth competition for survival between the herbivore and plant host (Kingsolver et al. 2012). Future studies may examine if the transgenerational effects of trichomes on offspring size, metabolism, and survival further benefit

the herbivores by accumulating advantages over subsequent generations. The role of familial lines in epigenetic inheritance may also be important to understand. For now, this study shows that non-glandular, stellate trichomes are able to trigger parental effects following consumption by the first generation. More broadly, this study serves as an example of the transgenerational effects of organismal damage.

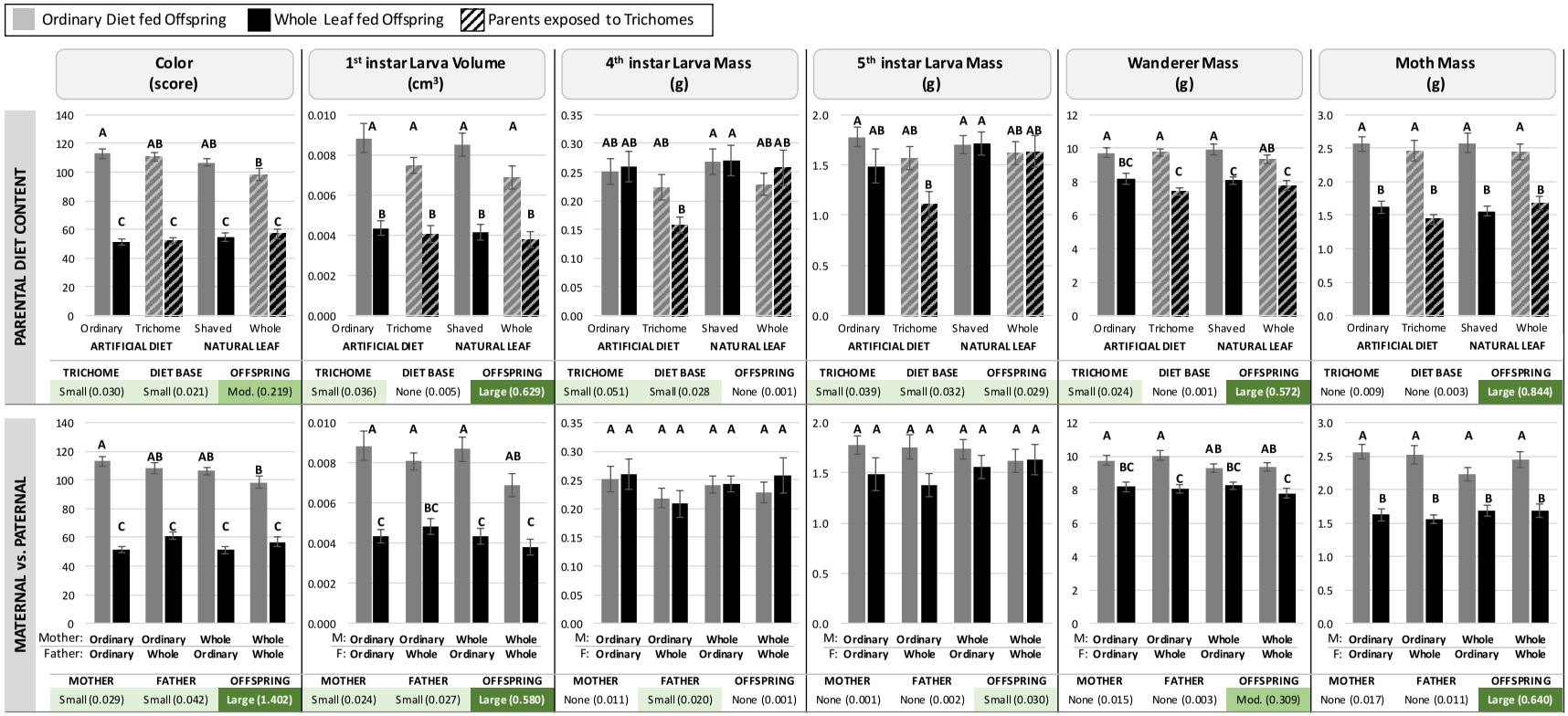
Ordinary Diet fed Offspring
Whole Leaf fed Offspring
Parents exposed to Trichomes
Color (score)
1st instar Larva Volume (cm3)
4th instar Larva Mass (g)
5th instar Larva Mass (g)
Wanderer Mass (g)
Moth Mass (g)
PARENTAL DIET CONTENT
MATERNAL vs. PATERNAL
Ordinary Trichome Shaved Whole
ARTIFICIAL DIET
NATURAL LEAF
Mother: Ordinary Ordinary Whole Whole
Father: Ordinary Whole Ordinary Whole
M: Ordinary Ordinary Whole Whole
F: Ordinary Whole Ordinary Whole
TRICHOME DIET BASE OFFSPRING
Small (0.030) Small (0.021) Mod. (0.219)
Small (0.036) None (0.005) Large (0.629)
Small (0.051) Small (0.028 None (0.001)
Small (0.039) Small (0.032) Small (0.029)
Small (0.024) None (0.001) Large (0.572)
None (0.009) None (0.003) Large (0.844)
MOTHER FATHER OFFSPRING
Small (0.029) Small (0.042) Large (1.402)
Small (0.024) Small (0.027) Large (0.580)
None (0.011) Small (0.020) None (0.001)
None (0.001) None (0.002) Small (0.030)
None (0.015) None (0.003) Mod. (0.309)
None (0.017) None (0.011) Large (0.640)

Figure 4-1: Comparison of color and size of offspring. Metrics are organized in columns from left to right: first instar color (scale from 0 (black) to 255 (white), first instar volume (cm^3), larval mass at fourth instar (g), larval mass at fifth instar (g), larval mass at wandering (g), moth mass at eclosion (g). Gray bars indicate offspring reared on Ordinary Diet; black bars indicate offspring reared on Whole Leaves; striped bars indicate parents were exposed to trichomes. Error bars indicate standard error. Different letters on bars indicate statistically significant differences between groups using a Tukey post hoc comparison. Effect sizes listed under each bar graph and were calculated using Cohen's f^2 (Small effect: $f^2 > 0.02$, Moderate effect: $f^2 > 0.15$, Large effect: $f^2 > 0.35$) (Cohen 1988). Top row compares the effects of trichomes and natural leaves consumed by the parents (1st instar: n = 197, 4th instar: n = 175, 5th instar: n = 166, Wanderer: n = 159, Moth: n = 135). Parents were fed an artificial diet without (Ordinary Diet) or with (Trichome Diet) trichomes added, or a natural diet (real leaves) without (Shaved Leaves) or with (Whole leaves) trichomes intact. Effect sizes for parental Trichome consumption, parental Diet Base Type, and Offspring Diet Type are listed under each bar graph. Bottom row compares the maternal and paternal contributions to offspring (1st instar: n = 211, 4th instar: n = 210, 5th instar: n = 200, Wanderer: n = 194, Moth: n = 161). Parental mating pairs are represented by pairs of bars on each graph from left to right: both parents Ordinary Diet; mother reared on Ordinary Diet, father reared on Whole Leaves; mother reared on Whole Leaves, father reared on Ordinary Diet; both parents reared on Whole Leaves. Effect sizes for maternal, paternal, and offspring diet are listed under each bar graph.

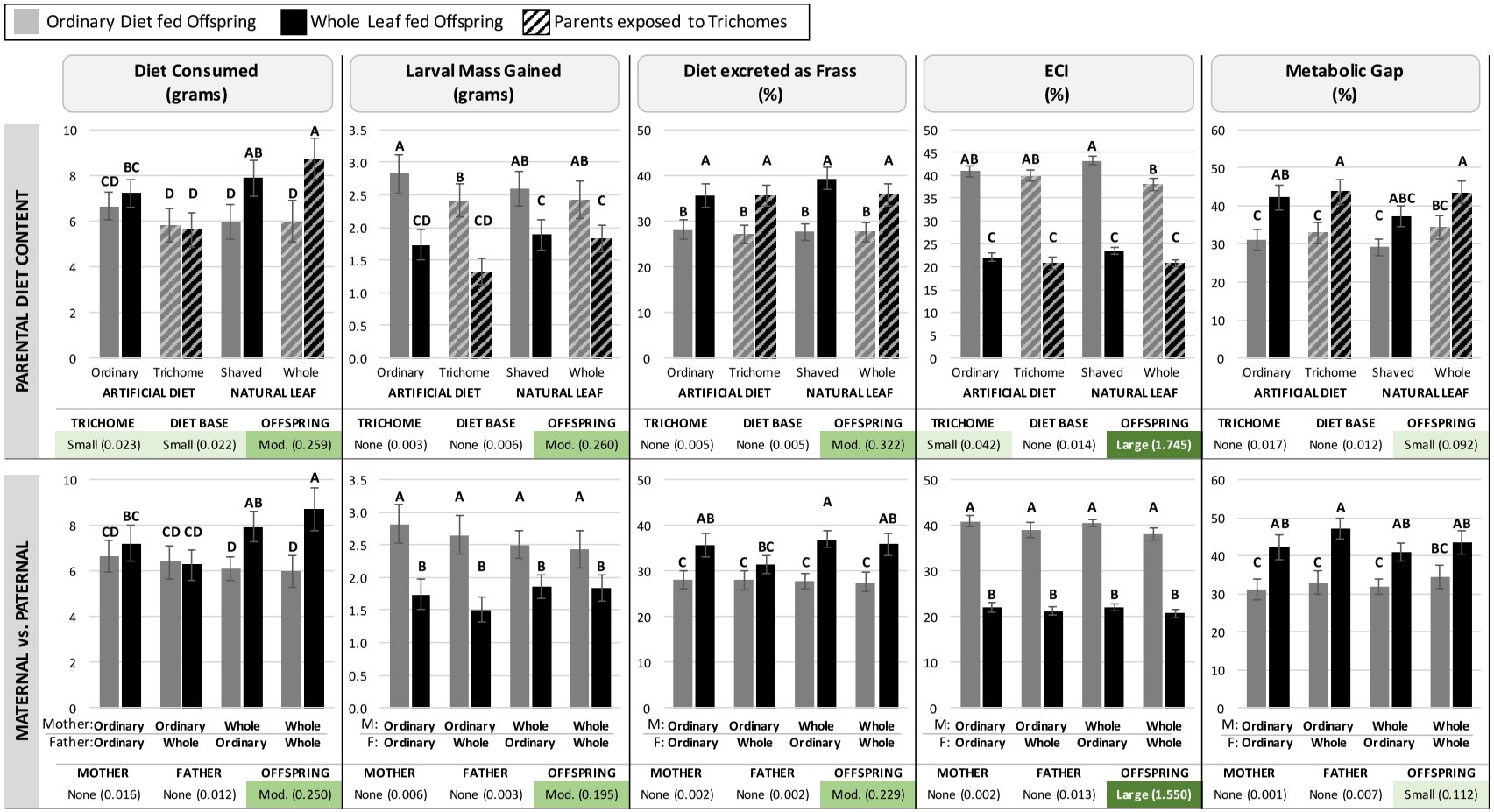
Ordinary Diet fed Offspring
Whole Leaf fed Offspring
Parents exposed to Trichomes
Diet Consumed (grams)
Larval Mass Gained (grams)
Diet excreted as Frass (%)
ECI (%)
Metabolic Gap (%)
PARENTAL DIET CONTENT
MATERNAL vs. PATERNAL
Ordinary
Trichome
Shaved
Whole
ARTIFICIAL DIET
NATURAL LEAF
Mother: Ordinary Ordinary Whole Whole
Father: Ordinary Whole Ordinary Whole
M: Ordinary Ordinary Whole Whole
F: Ordinary Whole Ordinary Whole
TRICHOME
DIET BASE
OFFSPRING
MOTHER
FATHER
Small (0.023)
Small (0.022)
Mod. (0.259)
None (0.003)
None (0.006)
Mod. (0.260)
None (0.005)
None (0.005)
Mod. (0.322)
Small (0.042)
None (0.014)
Large (1.745)
None (0.017)
None (0.012)
Small (0.092)
None (0.016)
None (0.012)
Mod. (0.250)
None (0.006)
None (0.003)
Mod. (0.195)
None (0.002)
None (0.002)
Mod. (0.229)
None (0.002)
None (0.013)
Large (1.550)
None (0.001)
None (0.007)
Small (0.112)

Figure 4-2: Comparison of diet consumption and efficiency metrics for fifth instar hornworm larvae after 48 hours of feeding on treatment diets. Metrics are organized in columns from left to right: mean amount of diet consumed (g), mean mass gain (g), percent of diet excreted as frass (%), mean efficiency of conversion (conversion efficiency, %), and mean metabolic gap (%). Gray bars indicate offspring reared on Ordinary Diet; black bars indicate offspring reared on Whole Leaves; striped bars indicate parents were exposed to trichomes. Error bars indicate standard error. Different letters on bars indicate statistically significant differences between groups using a Tukey post hoc comparison. Effect sizes listed under each bar graph and were calculated using Cohen's f^2 (Small effect: $f^2 > 0.02$, Moderate effect: $f^2 > 0.15$, Large effect: $f^2 > 0.35$) (Cohen 1988). Top row compares the effects of trichomes and natural leaves consumed by the parents (n = 341). Parents were fed an artificial diet without (Ordinary Diet) or with (Trichome Diet) trichomes added, or a natural diet (real leaves) without (Shaved Leaves) or with (Whole leaves) trichomes intact. Effect sizes for parental Trichome consumption, parental Diet Base Type, and Offspring Diet Type are listed under each bar graph. Bottom row compares the maternal and paternal contributions to offspring (n = 410). Parental mating pairs are represented by pairs of bars on each graph from left to right: both parents Ordinary Diet; mother reared on Ordinary Diet, father reared on Whole Leaves; mother reared on Whole Leaves, father reared on Ordinary Diet; both parents reared on Whole Leaves. Effect sizes for maternal, paternal, and offspring diet are listed under each bar graph.

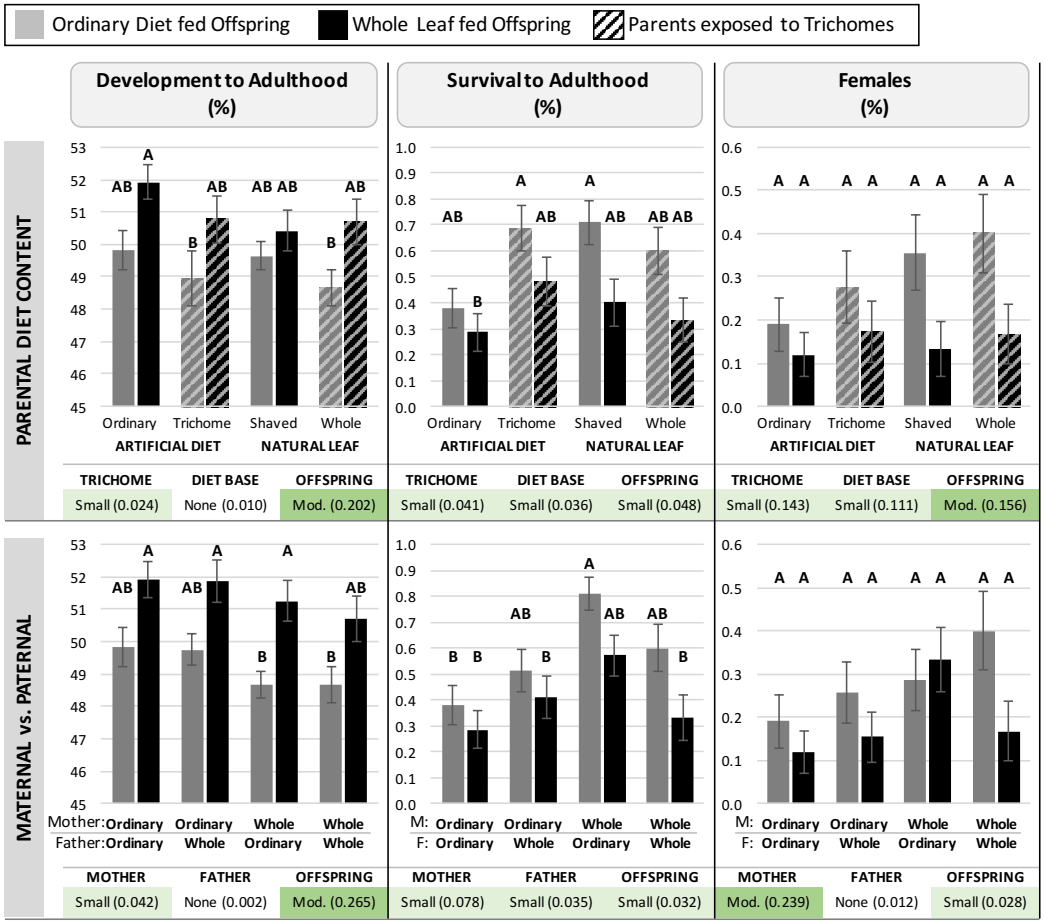

Figure 4-3: Comparison of development time, survival, and females of offspring. Metrics are organized in columns from left to right: development time from hatching to eclosion (days), survival from hatching to eclosion (%), proportion of adult females from the initial population (%). Gray bars indicate offspring reared on Ordinary Diet; black bars indicate offspring reared on Whole Leaves; striped bars indicate parents were exposed to trichomes. Error bars indicate standard error. Different letters on bars indicate statistically significant differences between groups using a Tukey post hoc comparison. Effect sizes listed under each bar graph and were calculated using Cohen's f^2 (Small effect: $f^2 > 0.02$, Moderate effect: $f^2 > 0.15$, Large effect: $f^2 > 0.35$) (Cohen 1988). Top row compares the effects of trichomes and natural leaves consumed by the parents (Development: n = 124, Survival: n = 263, Females: n = 263). Parents were fed an artificial diet without (Ordinary Diet) or with (Trichome Diet) trichomes added, or a natural diet (real leaves) without (Shaved Leaves) or with (Whole leaves) trichomes intact. Effect sizes for parental Trichome consumption, parental Diet Base Type, and Offspring Diet Type are listed under each bar graph. Bottom row compares the maternal and paternal contributions to offspring (Development: n = 150, Survival: n = 306, Females: n = 306). Parental mating pairs are represented by pairs of bars on each graph from left to right: both parents Ordinary Diet; mother reared on Ordinary Diet, father reared on Whole Leaves; mother reared on Whole Leaves, father reared on Ordinary Diet; both parents reared on Whole Leaves. Effect sizes for maternal, paternal, and offspring diet are listed under each bar graph.

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Appendix

Trichome Density Calculation

The following calculation and supporting validation experiments were performed in order to determine an ecologically relevant concentration of trichomes to include in the Trichome Diet treatment.

VALIDATION EXPERIMENTS

In order to determine the amount of trichomes to include in artificial diet, I began by measuring the amount of leaf tissue and the amount diet consumed in one hour. Larvae were reared from hatching in groups of ~50 larvae within rearing cups. Cups were assembled by pouring prepared Ordinary Diet (P/N: F9783B, Frontier Agricultural Science Newark, DE) into the base of 11.6 x 15.1 cm deli cups, lining them with a sheet of stiff plastic climbing mesh (P/N: VX620, Frost King by Thermwell, Mahwah, NJ), fitting them with aerated lids (P/N: D32CX, Anchor Packaging, Paragould, AR and P/N: FAB PPLID, Fabri-Kal, Kalamazoo, MI), and inverting them (so that the lid is now the base and the food sits at the ceiling) Larvae fed ad libitum on Ordinary Diet from hatching to fourth instar. After molting to fifth instar, 48 larvae were transferred to individual, 100 x 10 mm Petri dishes (P/N: 351029, Corning, Corning, NY) and provided either fresh horsenettle leaves or a cube of Ordinary Diet ad libitum for one hour. Initial mass of diet provided was measured to the nearest 0.001 g using an electronic pan balance and the mass of the remaining diet was measured after an hour. Leaves were digitally scanned prior to feeding and again after one hour. The amount of surface area consumed by larvae was determined by measuring the surface area using ImageJ and subtracting the amount of remaining surface area from the total initial surface area. These values allowed me to estimate the amount of diet larvae consumed.

The number of trichomes was previously measured to be approximately 1053 trichomes per square centimeter of horsenettle leaf (Kariyat et al 2013). Rather than count the number of individual trichomes incorporated in Ordinary Diet, this amount of trichomes was converted to a mass. Ten groups of 10 trichomes were measured using a torsion balance to determine an approximate trichome mass. This made it possible to calculate the mass of trichomes consumed in one hour. Together with the mass of Ordinary Diet consumed in one hour, it was possible to determine an ecologically relevant mass of trichomes to add to Ordinary Diet.

RESULTS & CALCULATION

In this study, newly-molted, 4th instar larvae consumed an average of 0.09 g of artificial diet per hour. In the same hour, a second set of newly-molted, 4th instar larvae consumed an average of 3.1 cm2 of horsenettle per hour. On average, a cm2 of a horsenettle leaf has 1053 trichomes (Kariyat et al. 2013). This equates to consuming an average of 3264 trichomes per hour. The mass of 10 trichomes was measured to be 0.405 ug, therefore 3264 trichomes have a mass of 132 ug. Together, this means 0.09 g of artificial diet should contain 132 ug of trichomes. Or, to use standardized units, 1 g of artificial diet should contain 0.001467 g of trichomes.

Number of Trichomes Consumed per Hour

$$\frac{3.1\ cm^2\ leaf}{1\ hour} \times \frac{1053\ trichomes}{1\ cm^2\ leaf} \times \frac{0.405\ ug}{10\ trichomes} = \frac{132\ ug}{1\ hour}$$

Number of per gram of Artificial Diet

$$\frac{132\ ug\ trichomes}{1\ hour} \times \frac{1000000\ ug}{1\ g} \times \frac{1\ hour}{0.09\ g\ Ordinary\ diet} = \frac{0.001467\ g\ trichomes}{1\ g\ Ordinary\ diet}$$

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Exploration U Instructor, multiple instances 2013 – 2018
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