FACTORS AFFECTING METABOLISM DURING NON-FEEDING STAGES IN INSECTS

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DOCTOR OF PHILOSOPHY

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ABSTRACT

Although feeding is important for optimal development and growth in insects, there are several points during the insect life cycle that are non-feeding: metamorphosis, pupation, and overwintering. Non-feeding periods also occur in response to internal cues, such as feedback from nutrient thresholds and immune responses being activated. Additionally, as an insect goes through different developmental stages, its nutritional requirements change in response to or in preparation for non-feeding periods. Most physiological responses like feeding are regulated through an interconnection of pathways, but how these networks change in response to different energy demands, such as immune challenges or changes in metabolism, is poorly understood. One significant pathway that is involved in regulating several physiological processes is the insulin signaling pathway. In my dissertation research, I tested hypotheses explaining the regulation of physiological processes during non-feeding periods in two agriculturally relevant insects, *Manduca sexta* and *Megachile rotundata*. First, I investigated how internal cues such as dietary lipid content and immune challenges cause non-feeding periods in *M. sexta*. Then, I investigated how insulin signaling regulates development during a non-feeding period like overwintering changes in *M. rotundata.* Since the insulin signaling (IIS) pathway is critical for development and growth, I focused on testing if this pathway plays a role in regulating non-feeding periods. My research showed that increased dietary lipid content causes a cessation of feeding, which suggests there is a possible lipid threshold that when reached, causes *M. sexta* to switch from lipid consumption for storage to lipid excretion. When looking at another cue like immune challenges, my results showed that during a bacterial infection, a Toll-mediated suppression of IIS pathway may be regulating feeding and causing a non-feeding period exhibited as sickness-induced anorexia. Lastly, my results also showed that the IIS pathway is

suppressed in overwintering *M. rotundata*, and that this process can change in response to temperature. Overall, my dissertation research showed that the insulin signaling pathway and nutrient content play a vital role in regulating non-feeding periods. Investigating insulin signaling, lipid metabolism, and innate immunity in these species closes a gap in knowledge of invertebrate development.

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DEDICATION

This PhD is dedicated to my family for all their sacrifices, patience, support, and help in getting my through these past six years. My motivation for this PhD always was my nieces and nephews, so this is to show them that 1) we can do whatever we sent our minds to, 2) we are not a product of our circumstances, and 3) hard work will always trump talent. I would also like to dedicate this to all the first generation Americans and students. Several of us started our journeys by putting ourselves out there and looking for opportunities on our own. We continuously fight for our dreams without a road map, so we create our own. This is also dedicated to all the other students who battle with their mental health every day. Your worth is not determined by your productivity. Your mental illness does not make you any less smart. Have patience with the journey and with yourself. And remember, there is always something to live for. Always.

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CHAPTER 1. REGULATION OF PHYSIOLOGICAL PROCESSES BY THE INSULIN SIGNALING PATHWAY IN INSECTS

Overview

The class of Insecta, or hexapod invertebrates, is the largest and most diverse group in the animal kingdom (Stork, 2018). With over 5 million predicted species, only approximately 20% have been identified and studied (Stork, 2018). Insect evolution was estimated to have occurred over 300 million years ago (Engel and Grimaldi, 2004; Stork, 2018). Insects live in a wide array of habitats that have seasonal changes. As ectotherms, insects primarily obtain heat from their environment and must respond to changes in seasons (Heinrich, 1995). Insects also have to be able to respond to their changing environments to survive. These behaviors and responses to their environment are governed by various complex and often interacting physiological systems and processes. Physiological systems interconnect and communicate to regulate the lifecycle and development of an insect. However, the mechanisms that coordinate the changes in these processes throughout the lifetime of an insect are still unclear for many systems. How does an insect change its food intake to meet requirements for larval growth versus reproduction? What mechanisms direct communication between systems? One putative factor is the insulin/insulin growth factor-1 signaling pathway (IIS), which is well known to regulate growth, development, and both carbohydrate and lipid metabolism in mammals. Given its importance in regulating physiological processes in both vertebrates and invertebrates (Cheetham and Brand, 2013; Claeys et al., 2002; Das and Dobens, 2015; Gronke and Partridge, 2010; Gu and Chen, 2009; Wu and Brown, 2006; Wu et al., 2004), IIS is one possibility. In this literature review, I describe the current knowledge of insect physiological processes, such as nutrient regulation, immunity, and development. I also include a background on the history of insulin signaling in insect

development and how it varies across taxa and life stage. I specifically describe how insulin-like peptides (ILP) are involved in nutrition and immunity across development, as well as the gaps in knowledge and questions remaining to be answered.

Insect development

Holometabolous life cycle

Insect life cycles can be hemimetabolous or holometabolous. Hemimetabolous insects undergo incomplete metamorphosis, with only three distinct stages: egg, nymph, and adult (Nation, 2015). Without a pupal stage, in which complete metamorphosis would occur, nymphs of hemimetabolous insects resemble adults but lack reproductive and flight capabilities (Gullan, 2005; Nation, 2015). Holometabolous insects, on the other hand, go through complete metamorphosis, with four stages, egg, nymph, pupa, and adult (Klowden, 2013; Nation, 2015). Holometabolous insects often have very different requirements for each life stage, inhabiting different environments and performing different activities (Klowden, 2013; Nation, 2015). For instance, larval insects are generally focused on feeding for growth and development (Zhu and Cutkomp, 1992). This means they may spend most of their time on their food source. In contrast, pupae are usually non-feeding and spend most of their time in protected sites like underground burrows. Burrowing is just one of many mechanisms insects use to protect themselves from predation and the surrounding environment. Another mechanism insects use to survive unfavorable conditions is to go through a developmentally dormant period, diapause, at any of the stages in their life cycle (Denlinger, 2002).

Diapause and overwintering

Insects living in tropical and temperate zones undergo a stage of developmental arrest, known as diapause, to survive environmental stressors, especially low temperatures of winter

(Hahn and Denlinger, 2011; Sim and Denlinger, 2013a; Yocum et al., 2015). Information about the environmental requirements for diapause, such as temperature, humidity, and photoperiod, has allowed commercially managed insects to be successfully stored during this stage. However, the exact control of diapause is still unknown, and the changes in seasons and temperature associate with climate change have made it even more important to better understand the regulation of diapause.

For many insects, diapause is a non-feeding stage, meaning all feeding to collect sufficient energy reserves for the winter and subsequent period of development must be done prior to diapause initiation (Denlinger, 2002; Hahn and Denlinger, 2007, 2011; Lehmann et al., 2016). Energy reserves not only help insects survive the winter but also provide the initial energy to insects to resume development and emergence after overwintering. Several insect species emerging from overwintering need to have energy to feed and prepare for reproduction (Hahn and Denlinger, 2011). Energy expenditure during overwintering is minimized by a decrease in metabolic rate, a key character indicating the onset of diapause (Hahn and Denlinger, 2011). The main energy reserves of diapausing insects consist of three groups of macronutrients, amino acids, carbohydrates, and lipids (Hahn and Denlinger, 2007, 2011). All three major macronutrients are required prior to diapause initiation, but it is unclear how the use of these nutrients is regulated.

During diapause, insects are at risk for environmental stressors, but depressed metabolism, a key characteristic of diapause, can lead to enhanced stress tolerance (Denlinger, 2002; Hahn and Denlinger, 2007, 2011; Sim and Denlinger, 2008; Wu and Brown, 2006; Wu et al., 2004; Zhou and Miesfeld, 2009). In the cotton bollworm, *Helicoverpa armigera* , hypoxia leads to an increase in production of reactive oxygen species, which in turn uses forkhead box

transcription factor (FOXO), a component of the insulin signaling pathway, to initiate signaling that promotes resistance to low temperature, oxidative stress, and pathogenic infections (Zhang et al., 2017). This cross-talk makes the IIS pathway a likely candidate for diapause regulation in insects (Antonova et al., 2012; Matsunaga et al., 2016; Schiesari et al., 2016; Sim and Denlinger, 2007; Wu and Brown, 2006; Wu et al., 2004), although the mechanism behind IIS regulation of diapause is unclear. Because of the fixed energy reserves overall metabolism and insulin signaling are maintained at low levels, but it is unclear if these change in response to stressors. I hypothesize that insulin signaling is responsible for allocating energy in response to stressors such as fluctuating temperatures during overwintering. Investigating how insects regulate diapause and how those mechanisms respond to stress is critical for understanding how climate change may impact insect development, especially for pollinators.

Insect nutrition

Nutrient requirements for development

Nutritionally-balanced diets are critical for proper development, reproduction, and growth in both vertebrates and invertebrates. Similar to vertebrates, insects' dietary requirements include major macromolecules: carbohydrates, lipids, proteins, amino acids, vitamins, and minerals (Raubenheimer, 1992; Raubenheimer and Simpson, 1996, 1997; Simpson and Raubenheimer, 1993). As an insect goes through different life stages, its nutritional requirements also change. The major focus of early larval stages is growth and development (Zhu and Cutkomp, 1992). For example, larvae of the tobacco hornworm *Manduca sexta*, prefer a diet of 2:1 protein to carbohydrate, which is optimal for growth and development (Thompson et al., 2001). However, during late larval stages, where the focus is to accumulate reserves for pupation

and metamorphosis, the high energy content of lipids may be more desirable (Fernando-Warnakulasuriya et al., 1988; Tsuchida and Wells, 1988).

Carbohydrates

Carbohydrates are the major energy source for insects (Thompson, 1999; Thompson and Dahlman, 1998). Carbohydrates are generally absorbed in the form of monosaccharides, requiring enzymes to break down poly- and disaccharides into monosaccharides (Klowden, 2013; Thompson, 1999). Insects show optimal growth when their diets have increased levels of carbohydrates (Chapman, 1998). However, use of excess carbohydrate content depends on the insect's ability to hydrolyze polysaccharides to oligosaccharides and its ability to absorb complex carbohydrates. One example is the polysaccharide cellulose, the main component of plant cell walls, which cannot be catabolized by most insects except termites and honeybees (Martin, 1983).

In preparation for metamorphosis, insects obtain their maximum carbohydrate intake as larvae. During metamorphosis, the main energy source is glucose, which is provided by glycogen stores in the fat body and transported through the blood as trehalose, the major hemolymph sugar in insects (Thompson, 2003). Glucose is also used for cuticle components, such as chitin. Furthermore, carbohydrates are necessary for male and female reproductive systems and embryonic development. Sugars form an important part of the testes and the seminal plasm, while the successful development of an embryo depends on the preparatory step of carbohydrate yolk accumulation in the oocyte (Klowden, 2013; Nation, 2015). In addition to their role in growth and reproduction, carbohydrates can be used as a readily mobilized substrate to initiate flight.

Lipids

Just like in many mammals and invertebrates, the high energy value of lipids is important for storage, development, and metamorphosis in insects (Beenakkers et al., 1985; Canavoso et al., 2001; Mirth and Riddiford, 2007; Nijhout et al., 2014; Ojeda-Avila et al., 2003). Fatty acids are the primary lipids in insects and are needed for hormone synthesis (sterols), cell membrane components (phospholipids), and a wide range of other physiological processes, such as immune responses (polyunsaturated fatty acids; PUFAs) (Stanley-Samuelson et al., 1988). Fatty acids are absorbed by the walls of the midgut lumen, transported by lipoproteins through the hemolymph as diacylglycerides (DAG), and stored in the form of triacylglycerides (TAG) (Arrese et al., 2010; Canavoso et al., 2001; Stanley-Samuelson et al., 1988; Turunen and Chippendale, 1977).

The importance of lipid transport and storage in insects is well understood, but very few studies have considered lipid metabolism and its role in other physiological processes, such as the immune response. During an immune challenge, lipid content can be important since PUFAs are needed for immune responses. Adamo *et al.* (2010) describe sickness-induced anorexia (SIA) as an energy reallocation, a trade-off from lipid transport to immune function. When given a high-fat diet, field crickets *Gryllus texensis* not only had a higher lipid concentration in their hemolymph, but also a lower survival against a bacterial infection (Adamo et al., 2010). Similar results are seen even without an immune challenge in *M. sexta*. When reared on a high-fat diet, fifth instar *M. sexta* were smaller, had delayed development, and exhibited smaller, more lipid-concentrated fat bodies (Cambron et al., 2019).

Amino acids

The third major group of macromolecules, amino acids are synthesized and stored mainly in the fat body (Arrese and Soulages, 2009; Keeley, 1985), main storage organ, and are needed

for the synthesis of other proteins and neurotransmitters, cuticle sclerotization, and for intermediate products in metabolic pathways. There are certain amino acids that are essential for insects, meaning they cannot be made and must be acquired through the diet (Davis, 1975). Synthesis of non-essential amino acids is achieved by transamination of amino groups from existing amino acids. The aromatic rings in phenylalanine are used to create tyrosine, an amino acid needed for cuticular sclerotization. Cysteine is another amino acid that must be derived from other sulfur-containing amino acids, like methionine, and has been shown to be needed for antifungal peptide production (Lamberty et al., 1999).

Nutrient regulation

To acquire the necessary nutrients, vitamins, and minerals, insects perform dietary self-selection, choosing a diet that has a higher content of the missing nutrient from a free choice of diets (Simpson and Raubenheimer, 1993; Thompson and Redak, 2000, 2005, 2008). Different developmental stages require different nutrients, making dietary self-selection an important mechanism for optimal growth and development (Raubenheimer and Simpson, 1999; Simpson and Raubenheimer, 1993). The mechanisms behind dietary self-selection and nutrient regulation in insects have interested scientists since the turn of the twentieth century yet questions about the mechanisms of nutrient sensing and regulation remain (Abisgold et al., 1994; Bernays and Bright, 1993; Busse and Barth, 1985; Fewell and Winston, 1996; Raubenheimer and Simpson, 1999; Siegert, 1988; Siegert and Ziegler, 1983; Siegert, 1995; Siegert and Mordue, 1992, 1994; Siegert et al., 1993; Simpson and Raubenheimer, 1993; Trumper and Simpson, 1993). Many studies point to the fat body and the IIS/TOR pathway as key players in these processes (Anand and Lorenz, 2008; Arrese and Soulages, 2009; Perez-Hedo et al., 2013; Povey et al., 2014; Thompson and Redak, 2000, 2005, 2008).

Role of the fat body

The insect fat body, a multifunctional tissue equivalent to vertebrate adipose tissue and liver in its storage and major metabolic functions, plays a key role in assessing nutritional status, via cues such as trehalose concentration and amino acid content (Anand and Lorenz, 2008; Arrese et al., 2010; Arrese et al., 2008; Azeez et al., 2014; Barrio et al., 2014). This mechanism, defined as nutritional signaling, also allows for a direct humoral relay from the fat body to the brain to adjust dietary preference (Povey et al., 2014; Thompson and Redak, 2000, 2005, 2008; Thompson et al., 2002; Thompson et al., 2001). In addition to nutrient regulation, the fat body also functions to store and release energy needed for insect development and reproduction. Fat body cells, known as adipocytes, store carbohydrates in the form of glycogen and lipids in the form of triacylglycerides (Arrese and Soulages, 2009). To be able to integrate nutritional cues and perform multiple metabolic functions, the fat body is the target of many hormones (Cerkowniak et al., 2015). Of those hormones, adipokinetic hormone (AKH) (Anand and Lorenz, 2008; Siegert and Mordue, 1994) and insulin-like peptides (ILP) (Bai et al., 2012; Keshan et al., 2017; Liu et al., 2010; Nilsen et al., 2011; Roy et al., 2007) are key players for insect development, growth, reproduction, and even immunity. The fat body relays nutritional information by having fat body cells signal to insulin-producing cells in the organ, via the target of rapamycin (TOR) pathway, to secrete insulin-like peptides (ILP) into the hemolymph (Gulia-Nuss et al., 2011; Mizoguchi and Okamoto, 2013; Wu and Brown, 2006; Wu et al., 2004).

TOR pathway

The target of rapamycin (TOR) pathway, also known as the nutrient sensing pathway, has been described as the key regulator in the control of metabolism and is well-conserved among all eukaryotes, from plants to flies to mammals (Hall, 2008; Oldham, 2011). The TOR protein is

composed of two multiprotein complexes, TORC1 and TORC2 (Dunlop and Tee, 2009; Hall, 2008; Laplante and Sabatini, 2009). Although both complexes are important, more is known about TORC1 in regards to function and regulation. Most of the important functions of the TOR pathway highlighted in this review are carried out through TORC1.

TOR is a serine/threonine kinase that integrates both intracellular and extracellular signals, and is interconnected downstream with the insulin signaling pathway. TOR signaling plays a role in autophagy, growth, and stress (Laplante and Sabatini, 2009; Oldham, 2011; Rohde et al., 2001), and regulates translation factors, such as ribosomal protein S6 kinase (S6K). TOR is regulated by cellular levels of amino acids that are communicated by growth factors like ILP (Foster and Fingar, 2010; Kim et al., 2008; Lamming and Sabatini, 2013; Teleman et al., 2008). For example, in the African malaria mosquito, *Anopheles gambiae*, the increase of amino acid content after a blood meal activates the TOR pathway to regulate vitellogenin; an important step for initiating egg development (Arsic and Guerin, 2008). This regulation of vitellogenin gene expression is shown in the mosquito *Aedes aegypti* (Gulia-Nuss et al., 2011; Hansen et al., 2005). TOR signaling also regulates synthesis of juvenile hormone in mosquitos (Perez-Hedo et al., 2013; Sim and Denlinger, 2008) and in the German cockroach *Blattella germanica* (Maestro et al., 2009). The relay of intracellular nutritional status is vital for growth, development, and reproduction insects (Colombani et al., 2003; Shiao et al., 2008; Treins et al., 2002).

Because both insulin and Toll signaling pathways both operate in the adipocytes of the fat body, the role of TOR signaling in innate immune responses is becoming better understood. In both *Drosophila melanogaster* and mammals, the insulin signaling pathway and innate immune system form a network. The conserved protein kinase B/phosphatidylinositol 3-kinase (AKT) pathway utilizes targets in the insulin pathway, including phosphoinositide 3-kinase (PI3K) and

TOR (Buchon et al., 2014; DiAngelo et al., 2009; Libert et al., 2008; Roth et al., 2018) to activate antiviral autophagy. Due to the importance of TOR signaling in regulating metabolic homeostasis, studies are focusing on the role of TOR in diabetes, obesity, heart disease, and other age-related diseases (Bhaskar and Hay, 2007; Broughton et al., 2005; Catania et al., 2011; Dunlop and Tee, 2009; Harrison et al., 2009; Kinghorn et al., 2016; Luong et al., 2006; Rulifson et al., 2002). With their highly conserved pathways, much can be learned from using insect models to study metabolic diseases and regulation.

Insect immunity

Innate immunity

Insects lack antibody-mediated immune responses (adaptive immunity). However, the innate immune systems of insects effectively fight pathogens through both humoral and cell-mediated responses like those in vertebrate innate immune systems (Eleftherianos et al., 2006; Strand, 2008; Tanaka and Yamakawa, 2011). Cell-mediated immune responses are carried out by hemocytes and consist of phagocytosis, encapsulation, and nodule formation (Booth et al., 2015; Eleftherianos et al., 2006; Honti et al., 2014; Jiang et al., 2010; Marmaras and Lampropoulou, 2009; Tanaka and Yamakawa, 2011; Wilson-Rich et al., 2008). Humoral immune responses consist of production of anti-microbial peptides (AMP) (Strand, 2008; Tanaka and Yamakawa, 2011) and cytotoxic molecules such as phenoloxidase (PO) (Kanost et al., 2004; Tanaka and Yamakawa, 2011) and take place in the hemolymph and the fat body. Immune systems in vertebrates and invertebrates can have trade-offs or be influenced by other physiological processes (Demas and French, 2011; McCallum and Trauth, 2007; Tobler et al., 2015). For example, an insect's immunity can be impacted by its development stage (Benesova et al., 2009; Booth et al., 2015; Eleftherianos et al., 2006; Lu et al., 2006; Tian et al., 2010). In tobacco

hornworm caterpillars (*M. sexta*) infected with bacteria, younger insects had delayed development, decreased growth rates, and higher bacterial loads (Booth et al., 2015). Of these changes, delayed development resulted from decreased growth, likely due to sickness-induced anorexia (SIA), a response that has also been shown in *M. sexta* to parasitic infection and entomopathogenic bacteria (Adamo, 2005; Adamo et al., 2007).

Sickness-induced anorexia

Most animals stop feeding when their bodies become infected with pathogens; a condition called sickness-induced anorexia (SIA) (Adamo, 2005; Adamo et al., 2007; Mason et al., 2014; Povey et al., 2014). The mechanism behind SIA is still unknown, but is hypothesized to be an adaptive function that causes reallocation of energy towards immune function (Adamo, 2005; Adamo et al., 2010; Adamo et al., 2007; Mason et al., 2014; Povey et al., 2014; Singer et al., 2014). Ensuring that the immune system has enough resources to resist or fight infection could enhance survival. Even with clinical and experimental evidence of SIA, the underlying function and mechanism behind SIA and its role in immunity remains unresolved (Adamo et al., 2010; Adamo et al., 2007; Bluthe et al., 2006; Dantzer and Swanson, 2012; Dantzer, 2001a, b, 2004; Dantzer and Kelley, 2007; Faggioni et al., 1997; Kelley et al., 2003; PlataSalaman, 1996, 1997; Povey et al., 2014). In a study on baculoviral infection in the African armyworm *Spodoptera exempta*, individuals exhibited SIA followed by dietary self-selection, choosing diets with higher protein content from a free choice of diets (Povey et al., 2014). Although SIA could be beneficial by reallocating energy from digestion to immunity, cessation of feeding could also become harmful to the organism over time (Adamo et al., 2010; Booth et al., 2015; Langhans, 2007). Because insulin signaling is known to regulate feeding in many animals, insulin signaling may play a role in regulating SIA.

Insulin signaling pathway

Conservation between vertebrates and invertebrates

The insulin/insulin-like growth factor signaling (IIS) pathway plays a role in regulating development, growth, metabolism, and reproduction in animals from varying taxa (Bates et al., 2013; Cheng et al., 2005; Das and Arur, 2017; Das and Dobens, 2015; Dupont and Holzenberger, 2003; Dupont and Scaramuzzi, 2016; Kuczkowski and Brinkkoetter, 2017; Nässel et al., 2013; Nijhout et al., 2014; Tatar et al., 2003). Insulin signaling has been shown to be conserved between vertebrates and invertebrates (Aslam et al., 2011; Brogiolo et al., 2001; Claeys et al., 2002; Das and Dobens, 2015; Gu and Chen, 2009; Wu and Brown, 2006; Wu et al., 2004), containing the same core components: insulin receptor (InR), insulin-like peptides (ILP), insulin receptor substrate (IRS), phosphoinositide 3-kinase (PI3K), protein kinase B (PKB, also known as AKT and the forkhead box transcription factor (FOXO) (Fig. 1). In insects, insulin-producing cells receive cues via the TOR pathway to secrete insulin-like peptides (ILP) into the hemolymph (Gulia-Nuss et al., 2011; Mizoguchi and Okamoto, 2013; Wu and Brown, 2006; Wu et al., 2004). A key difference in vertebrate IIS is that they have only one or two genes for insulin, (Dumonteil and Philippe, 1996; Owerbach et al., 1980; Steiner et al., 1985), whereas the number of genes for insulin-like peptides (ILP) in insects can be highly variable across species (Antonova et al., 2012; Claeys et al., 2002; Riehle and Brown, 2002; Smith et al., 1997; Wu and Brown, 2006). For example, the number of ILP genes ranges from seven in *D. melanogaster* (Arquier et al., 2008; Brogiolo et al., 2001; Gronke et al., 2010; Gronke and Partridge, 2010) to 38 in the silkworm, *Bombyx mori* (Aslam et al., 2011; Kondo et al., 1996).

Insulin-like peptides

The very first insulin-like peptide, bombyxin, was isolated from the silkworm *Bombyx mori* (Adachi et al., 1989; Aslam et al., 2011; Kondo et al., 1996). Since its discovery, several studies have elucidated physiological functions of bombyxins, such as regulating nutrient-dependent growth (Mizoguchi, 1994; Mizoguchi and Okamoto, 2013), fat body development and function (Liu, 2010), and ecdysone receptor expression (Keshan et al., 2017), tying insulin signaling to metamorphosis and development in *B. mori*. Some roles of ILP are conserved between species, but others such as regulation of ecdysone expression are not conserved even within the same order of insect (Smith et al., 2014).

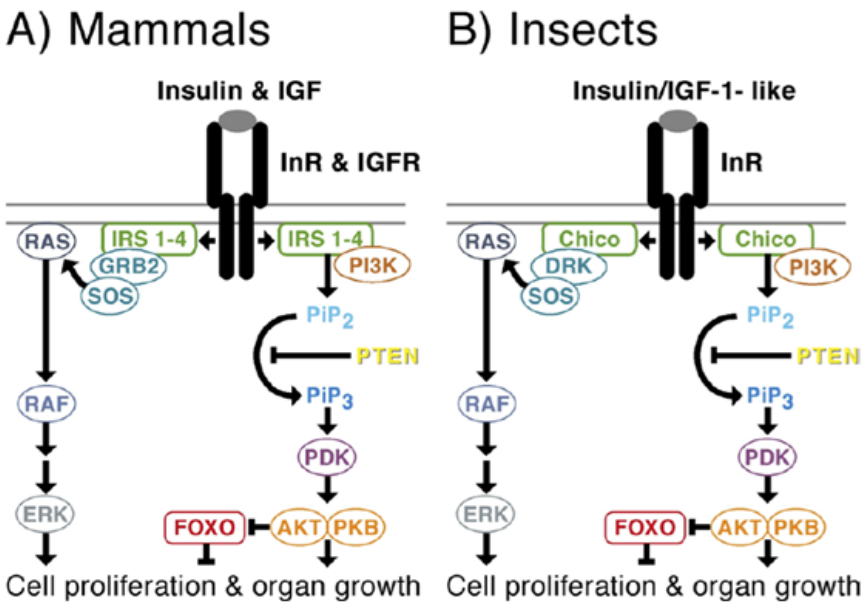

Figure 1. Comparison of core components of the insulin/IGF signaling between mammals and insects (Emlen et al., 2012).

Since the discovery of bombyxin, genes for ILP have been identified in species from Orthoptera, Diptera, Lepidoptera, and Hymenoptera, but their physiological functions are largely unknown (Aslam et al., 2011; Mirth et al., 2014; Mizoguchi and Okamoto, 2013; Nassel, 2012; Nässel et al., 2013; Nassel and Vanden Broeck, 2016; Wu and Brown, 2006). Most ILP bind to

only one receptor, adding to the complexity of understanding the signaling mechanism behind the various genes for ILP (Garofalo, 2002; Garofalo and Rosen, 1988; Kremer et al., 2018). Perhaps the most studied, functions for some of the ILP in the fruit fly *D. melanogaster* have been described, such as regulating growth rate and body size (Brogiolo et al., 2001; Rulifson et al., 2002), development (Britton et al., 2002), and lifespan (Buch et al., 2008; Rulifson et al., 2002). The number of genes for ILP is species-specific and can also vary by developmental stage (Bollenbacher et al., 1997; Kanost et al., 2016; Satake et al., 1997). Insulin-like peptides are expressed primarily in the brain and fat body of insects (Adachi et al., 1989; Iwami et al., 1996; Smit et al., 1998; Veenstra, 1989; Wu and Brown, 2006). However, tissue-specific insulin signaling varies throughout development. Individual components like ILP and InR have been shown to change throughout development in a tissue-dependent manner (Bollenbacher et al., 1997; Chen et al., 1996; Fernandez et al., 1995; Garofalo and Rosen, 1988; Kanost et al., 2016). For example, the 26 variants of ILP in *M. sexta* all show different expression patterns throughout the life cycle and within tissues (Bollenbacher et al., 1997; Kanost et al., 2016). In the brain, two ILP, ILP-Y and ILP-Z had the highest expression across all developmental stages (Kanost et al., 2016). However, in the adult midgut, all ILP showed similar expression levels (Kanost et al., 2016).

Similarly, development affects expression of the InR in *D. melanogaster*. During mid-embryogenesis, InR expression levels are high in embryonic epidermis (Fernandez et al., 1995), whereas in larvae InR expression levels are highest in imaginal discs (Chen et al., 1996). Another shift is seen in adult females, where InR levels are highest in ovary nurse cells (Garofalo and Rosen, 1988). Further developmental studies on ILP have shown they play an important role in diapause phenotypes, such as halted reproduction (Sim and Denlinger, 2009), reserve

accumulation (Satake et al., 1997), metabolic depression (Hahn and Denlinger, 2011), and enhanced stress-tolerance (Matsunaga et al., 2016; Sim and Denlinger, 2013b; Wu and Brown, 2006).

Significance of research

Insects are the largest taxa with the most biodiversity, as well as the group in the animal kingdom that we know the least about (Stork, 2018). Insects can cause around 20% of major crop losses, contributing to limitations on food production (Davies, 2014; Ferry et al., 2006). However, they also contribute to food production by pollinating crops such as alfalfa, blueberries, and pumpkin (Garibaldi et al., 2014; Kemp and Bosch, 2001; MacKenzie et al., 1997; Martin and McGregor, 1973; Minarro et al., 2018) (Pitts-Singer and Cane, 2011). Because of our reliance on insects for crop pollination, it benefits us to know as much as we can about their basic physiological processes in order to predict how climate change will impact them.

Whether a crop pest or pollinator, climate change is predicted to impact agriculturally-relevant insects and their development (Giannini et al., 2017; Memmott et al., 2007). In addition to changes in climate, other factors such as habitat loss, pesticides, predation, and pathogens, are putting many species at risk of extinction (Sánchez-Bayo and Wyckhuys, 2019). With temperature being one of the cues for insect development, understanding how insect physiology will be impacted by the environmental changes associate with climate change is critical for insect conservation.

In addition to being agriculturally important, insects have conserved physiological pathways that allow us to translate our findings from insect models to vertebrate studies. Clarifying what mediates SIA in insects would have a broad impact on our understanding of nutritional, immunological, and developmental biology in both vertebrates and invertebrates.

Furthermore, insect models are more cost-effective than traditional mammalian models, having shorter life cycles, which allows for more studies and faster results to advance our knowledge of physiology (Kavanagh and Reeves, 2004; Mylonakis and Aballay, 2005; Scully and Bidochka, 2006). Understanding components of insect physiology like insulin signaling, lipid metabolism, and innate immunity is important for improving our knowledge of invertebrate development. By focusing on the components of insect physiology that are conserved with vertebrates, we can work towards closing the gaps in knowledge in vertebrate health.

Objectives

Objective 1: Investigating the effects of dietary fat on *M. sexta* development

Nutritionally balanced diets are important for development, but for insects, fat is vital for its high-energy value (Arrese and Soulages, 2009; Birse et al., 2010; Fernando-Warnakulasuriya et al., 1988). In this study, I investigated how high-fat diets affect developing insects. To determine how increased dietary lipid affects insect growth and development, we used *M. sexta*, the tobacco hornworm caterpillar. Previous work on fifth instar *M. sexta* fed a high-fat diet showed delayed development and attainment of a similar body mass compared to those fed a control diet with a low level of fat (Fernando-Warnakulasuriya et al., 1988). Because caterpillars require energy to be stored for use during the adult stage, and lipids are a high-value form of energy storage, I hypothesized that high-fat diets positively affect insect development by achieving reserve stores quicker. Furthermore, I predicted that increased levels of dietary lipid would speed up development.

Objective 2: Studying the role of insulin signaling in mediating SIA in *M. sexta*

Because insulin signaling is well conserved from vertebrates to invertebrates and regulates feeding in humans (Schwartz et al., 2000), squirrels (Melnyk, 1981), rats (Prasad et al.,

1992), and dogs (Grossman et al., 1947), I hypothesized that the insulin signaling pathway also plays a role in SIA in *M. sexta*. To test the hypothesis that insulin signaling mediates SIA, I established a model of SIA with a nonpathogenic strain of *Escherichia coli* and methods for assessing SIA using body mass, fecal pellet mass and number, and food consumption in *M. sexta*. Using these methods, I examined the role of insulin signaling by either inhibiting or increasing insulin signaling using the pharmaceutical drug wortmannin or bovine insulin, respectively. To determine which genes may be involved in SIA, I measured expression of genes in the insulin signaling pathway and immunity-related genes.

Objective 3: Examining the impacts of temperature on insulin signaling gene expression in overwintering *Megachile rotundata*

Insects undergo a stage of developmental arrest, known as diapause, to survive environmental stressors, especially low temperatures of winter (Hahn and Denlinger, 2011; Sim and Denlinger, 2013a; Yocum et al., 2015). The alfalfa leafcutting bee, *Megachile rotundata*, is a commercially-managed, solitary pollinator that undergoes diapause as a prepupa. I investigated 1) how the insulin signaling pathway changes during overwintering and 2) how signaling changes in response to environmental stress such as fluctuating temperatures. I hypothesized that insulin signaling is involved in allocating energy in response to fluctuations in temperature during overwintering months. To test this hypothesis, alfalfa leafcutting bees, *M. rotundata*, were overwintered in either a lab setting at a constant 4˚C or in the field in naturally fluctuating temperatures, and then gene expression of the insulin pathway was measured. I predicted that bees overwintered in the field would have more variation in gene expression in response to naturally fluctuating temperatures.

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CHAPTER 2. EFFECTS OF HIGH-FAT DIET ON FEEDING AND PERFORMANCE IN THE TOBACCO HORNWORM, *MANDUCA SEXTA*[1]

Abstract

Nutritionally balanced diets are important for overall fitness. For insects, fat is vital for development due to its high-energy value. Little is known about how insects regulate dietary fat for storage, but research has shown conflicting results on how altering fat impacts development and performance. In this study, we sought to investigate how high-fat diets affect developing insects. To determine how insects respond to variation in dietary fat content, we reared *Manduca sexta* of different larval stages on diets containing varying concentrations of linseed oil in high (5.6%), medium (3.4%) or low (0.4%) fat. Young larvae reared on high-fat diets had 80% mortality and 43% lower body mass compared to those reared on medium- or low-fat diets. Older larvae showed no difference in mortality with increasing dietary fat content, but they were smaller than controls, suggesting a developmental shift in lipid metabolism. We measured mRNA expression of Apolipoprotein I and II (APO1 and 2), proteins responsible for transporting lipids, as a possible explanation of increased survival in older larvae. Levels of APO1 and 2 mRNA did not differ with dietary fat content. We then tested the hypothesis that the high-fat diet altered feeding, resulting in the observed decrease in body size. Caterpillars fed a high-fat diet indeed ate less, as indicated by a decrease in food consumption and the number and mass of fecal pellets produced. These results suggest that increased fat disrupted feeding and may indicate that

[1] The material in this chapter was co-authored by Lizzette D. Cambron, Gita Thapa, and *Kendra J. Greenlee. Gita and Lizzette had primary responsibility for collecting samples. Lizzette was the primary developer of the conclusions that are advanced here. Lizzette also drafted and revised all versions of this chapter. Dr. Greenlee served as proofreader and checked the math in the statistical analysis conducted by Lizzette.

there is a threshold for lipid storage, but further studies are needed to understand the underlying mechanism.

Keywords: insect; anorexia; nutrition; growth; development; juvenile; fat body; lipid content; high-fat diet

Introduction

Carbohydrates, proteins, nucleic acids, and lipids are the four macromolecules required for cellular function. Lipid, in particular, is a critical component of cell membranes and an important and efficient way for organisms to store energy. Obtaining a diet with balanced macronutrients is important for proper development and reproduction in all animals, including insects.

One strategy for insects to acquire adequate nutrition is to feed on different plant species or parts of the host plant (Waldbauer et al., 1984). Herbivorous animals, especially insects that eat only the vegetative parts of plants, may be taking in lower levels of dietary lipid if only consuming one part of the plant. *Hyalophora cecropia* larvae, which feed on a single plant species, mix their diets for optimal nutrient intake by eating different parts of the plant (Scriber, 1977). Plant seeds are typically high in lipid, but the leaves and stems contain lower levels of lipid (Durrett et al., 2008; Wang et al., 2007). An analysis of the nutritional content of *Datura innoxia*, one of the host plants of *Manduca sexta*, revealed that different parts of the plant had different levels of nutrients (Ayuba et al., 2011). The seeds contain 15.52% lipid and 13.9% protein, while the stem, pod and root contain 6% lipid and 2.9% protein. Another strategy to acquire balanced nutrients is to self-select a diet that maximizes growth and development.

Dietary self-selection is not only induced by imbalanced nutrients but several other factors like hemolymph trehalose levels and parasitization. Since Bell and Joachim (1976)

described methods for easily rearing *M. sexta* larvae in the laboratory on a wheat germ-based, artificial diet, experiments manipulating individual nutrients have helped in establishing nutritional requirements of *M. sexta*. Normal *M. sexta* larvae self-select a diet of 2:1 protein to carbohydrate, while parasitized larvae showed a preference of protein to carbohydrate ratios of 1:1 (Thompson et al., 2001).

In addition to eating various parts of a plant or self-selecting a diet to obtain sufficient lipid storage, insects can also synthesize lipids from carbohydrates for storage. When reared on a low-sucrose diet, *M. sexta* larvae deposited less fat in the body, but overall growth was not affected (Ojeda-Avila et al., 2003). Similarly, increasing carbohydrate content in the diet increased the fat content of the whole insect (Thompson et al., 2003; Warbrick-Smith et al., 2006). Lipid storage can also increase in response to changes in protein consumption (Ojeda-Avila et al., 2003; Schilder and Marden, 2006; Thompson et al., 2003; Warbrick-Smith et al., 2006). However, lipid synthesis can be energetically costly (Noreika et al., 2016), suggesting that acquisition from dietary sources should be preferred although studies have shown otherwise (Wilder et al., 2016). Studies have investigated the effect of manipulating dietary fat in insects (Birse et al., 2010; Fernando-Warnakulasuriya et al., 1988; Heinrichsen and Haddad, 2012; Stockhoff, 1993; Thompson, 2004), but these results showed conflicting effects on larval growth and development. Further studies in *M. sexta* are needed to better understand how altered dietary lipid impacts the regulation of other macronutrients, as well as the effects of dietary lipid on development and performance.

Because caterpillars require energy to be stored for use during the adult stage, and because lipid is a high-value form of energy storage, we reasoned that increased levels of dietary lipid should speed development. To determine how increased dietary lipid affects insect growth

and development, we used *M. sexta*, the tobacco hornworm caterpillar. Previous work on fifth instar *M. sexta* fed a high-fat diet showed delayed development and attainment of a similar body mass compared to those fed a control diet with a low level of fat (Fernando-Warnakulasuriya et al., 1988). We first fed caterpillars of various instars high-fat or low-fat diets. In contrast to previous findings, caterpillars in our studies fed high fat diets were smaller. To determine the mechanism for the decrease in body mass, we further explored lipid intake, transport, storage, and excretion in fifth instar caterpillars.

Materials and methods

Animal rearing

Manduca sexta larvae were reared from eggs at North Dakota State University (Fargo, ND, US) or purchased from Carolina Biological Supply (Burlington, NC, US). Hatched larvae were given ad libitum access to an artificial wheat germ-based diet and maintained under 16L:8D h at 25^{o} C as previously described (Vishnuvardhan et al., 2013).

Experimental diets

Experimental diets were prepared with increasing levels of fat, as previously done (Fernando-Warnakulasuriya et al., 1988). Variation in lipid content was achieved by changing the volume of corn oil or linseed oil per 1000 ml of diet (5.6%) HF: 30 ml, medium-fat (3.4%) MF: 17 ml, or low-fat (0.4%) LF: 4 ml). The volumes of water in the HF and MF diets were reduced to match the final volume of the LF diet. The low-fat (LF) diet was the standard laboratory diet for *M. sexta*. Corn oil was used for the first of our experiments, which resulted in poor growth of the LF diet-fed caterpillars compared to diets made using linseed oil, so we switched to linseed oil for the remainder of the experiments. Diets were not isocaloric, and the additional oil added 105 and 211 kilocalories per liter to the MF and HF diets, respectively.

Effects of high-fat diet on growth

To determine the effects of dietary fat on development throughout ontogeny, we fed caterpillars of different larval stadia diets with varying amounts of lipid. In the first experiment, upon hatching, first instar larvae were placed on LF, MF, or HF diets made with corn oil (n = 20 per group). In all other experiments, larvae were reared on LF food, the standard laboratory diet until they reached either the second (n =7 per diet), fourth (n =8 per diet), or fifth instar (n =10 per diet), at which time they were placed on the assigned experimental diet. Caterpillars were weighed daily and growth rates were calculated. For fourth instar larvae, we also noted the date of appearance of head capsule formation for molting. Additionally for fifth instar larvae, we noted the date of dorsal vessel appearance indicating the onset of wandering.

Food consumption

To determine whether the diet affected feeding, we measured food consumption in fifth instar caterpillars. Freshly molted fifth instar larvae (n = 10 per diet) were given 6 g of LF, MF or HF diet. After 24 hours, the remaining food was weighed to the nearest 0.1 g, and caterpillars were given a fresh, 6 g block of food. Food consumption was corrected for water loss, which was approximately 1% of food mass over 24 hours (0.18 ± 0.04 g).

Apolipoprotein mRNA expression

To determine whether changes observed in lipid content were due to alterations in lipid transport to tissues, we measured apolipoprotein mRNA expression from caterpillars in each treatment group. Freshly molted fifth instar caterpillars were given ad libitum access to a block of LF, MF, or HF diet (n = 9 per diet). Fat bodies were dissected from caterpillars from each diet treatment on days two, three, and four (n = 3 per day) of being on the treatment diets and stored in -80^{o}C for subsequent RNA extraction.

Total RNA was extracted from each fat body using TRIzol ® Reagent (Life Technologies, Grand Island, New York), following the manufacturer's protocol, except that the centrifugation was done at room temperature. We removed DNA contamination from total RNA (Ambion® TURBO™ DNase; Life Technologies, Grand Island, New York) and determined RNA quality by denatured agarose (1.2%) gel separation. One µg of RNA was used to synthesize cDNA (BIO-RAD iScript™ cDNA Synthesis Kit; Bio-Rad Inc., Hercules, California) in a reverse transcription reaction of 20 µl. Complementary DNA was diluted 10-fold with 1 mmol EDTA and 10 mmol Tris-HCl. Three biological replicates were used to run real-time quantitative-PCR (qRT-PCR). Reactions were carried out in a total volume of 20 µl with 1x Fast SYBR green master mix, 2 µl of diluted cDNA and 5µM qRT-apo-F2: 5'-TTCACTACAGAGGAGTCGAAC -3', qRT –apo-R2: 5'-GAGTGTCCAGGTCCTTCTTC-3' under standard cycling conditions. We designed primers based on the sequences for APO I and II (Gen Bank Accession number U57651) using Gene Fisher 2 software (Giegerich et al., 1996). Absolute copy numbers of APO I and II were calculated from a standard curve made from serial dilutions of (108) APO I and II transcripts.

Lipid content of fat body and fecal pellets

To determine whether HF diet-fed caterpillars store or excrete excess fat, we measured the lipid content of the fat body and fecal pellets. Freshly molted, fifth instar caterpillars were placed on LF, MF, or HF diet (n = 10 per diet). Fecal pellets were collected daily within a period of 24 h, until the fourth day of the fifth instar. On the fourth day, fat bodies were dissected out. Lipid content of fecal pellets and fat bodies were determined gravimetrically using a modified Folch's method for lipid extraction (Folch et al., 1957). We standardized the assay among samples by using the same amount of fat body tissue (0.1 g) or fecal pellets (0.15 g) for lipid

extraction per individual. Fecal pellets and fat body samples were dried to a constant mass before lipid extraction. Dried material was mechanically homogenized in a 2:1 v/v mixture of chloroform and methanol. The resulting solution was filtered, and the resulting filtrate was rinsed with 400 µl of 0.73% NaCl solution and allowed to sit until a biphasic state was achieved (30 - 60 min). The sample was then washed with 2 µl of a solution of 8:4:3 chloroform, methanol, and magnesium chloride. Solvent was evaporated for 48 hours, and then samples were washed with 0.33 ml chloroform and transferred to preweighed 10 ml vials. The samples were left overnight to let the chloroform evaporate and then reweighed. The lipid fraction was the difference between weights of the pre and post-chloroform removal.

Approximate digestibility

To further examine the decreased body mass of HF-fed caterpillars, we calculated the approximate digestibility (AD) of the three diets to determine if digestibility of the diet affected mass gain. Fifth instar caterpillars (n = 10 per diet) were given 7 g of HF, MF, or LF food daily for four days. After 24 h, leftover food was weighed and transferred to a pre-weighed 42 ml glass vial and then dried overnight in an oven at 56°C. Once dried, the 7 g aliquots were weighed again. We calculated approximate digestibility using the following equation

$$AD\ (\%) = 100\ (E - F) / E \qquad \text{(Eq. 1)}$$

where E = average dry weight of food eaten from each individual and F = average dry weight of feces produced for each diet (Reynolds and Nottingham, 1985). Approximate digestibility data were arc sine transformed before they were statistically analyzed.

Data analysis

IBM SPSS (versions 19 and 22) was used for statistical analysis. Data are presented as means ± S.E.M. throughout. This experiment was repeated in two different years. Because of

year-to-year variation in some measured variables (body mass and growth rate), we were unable to pool the results across years. For those measures, we present both sets of data. However, the effect of diet was the same in both years. To determine if body mass, growth rate and food consumption varied among diet treatments, we used repeated measures of analysis of variance (RM-ANOVA), with diet as the between subjects factor and time as the within subjects factor. Two-way ANOVA was used for the analysis of mRNA expression of apolipoprotein, with diet and time as factors. P values less than 0.05 indicated significant differences among means. Bonferroni-corrected post hoc tests were used to determine which means differed among the groups. Data were tested for normal distribution and for homogeneity of variances using Levene's test. Data that did not meet those assumptions were tested using the non-parametric, Jonckheere-Terpstra Test for Ordered Alternatives. To better interpret the possibility of food consumption being regulated by fat content, we analyzed tested for correlations between mass-corrected total protein consumption in fifth instar larvae versus non-protein consumption using an analysis of covariance (ANCOVA).

Results

Effects of high-fat diet on growth

Caterpillars fed a HF diet made with corn oil had higher mortality compared to those fed a LF diet (Fig. A1, Log Rank test, $\chi^2 = 8.36$, $p < 0.01$). The effect of the diet on growth depended on how many days a caterpillar had been feeding on it (Fig. A1; diet x day interaction, lower-bound adjusted $F_{1,18} = 21.29$, $p < 0.001$). When dietary fat was manipulated with linseed oil, and experimental diets were given at the beginning of the first, second or fourth instars, HF diet-fed caterpillars had significantly lower body masses (first instar (Fig. 2A): $F_{2, 15}=16.17$ P <

0.001; second instar (not shown): $F_{2, 21}$=13.63 P < 0.001; and fourth instar (Fig. 1B): $F_{2, 26}$=5.39 P < 0.025) and growth rates (Table 1) than compared to larvae fed MF and LF diets.

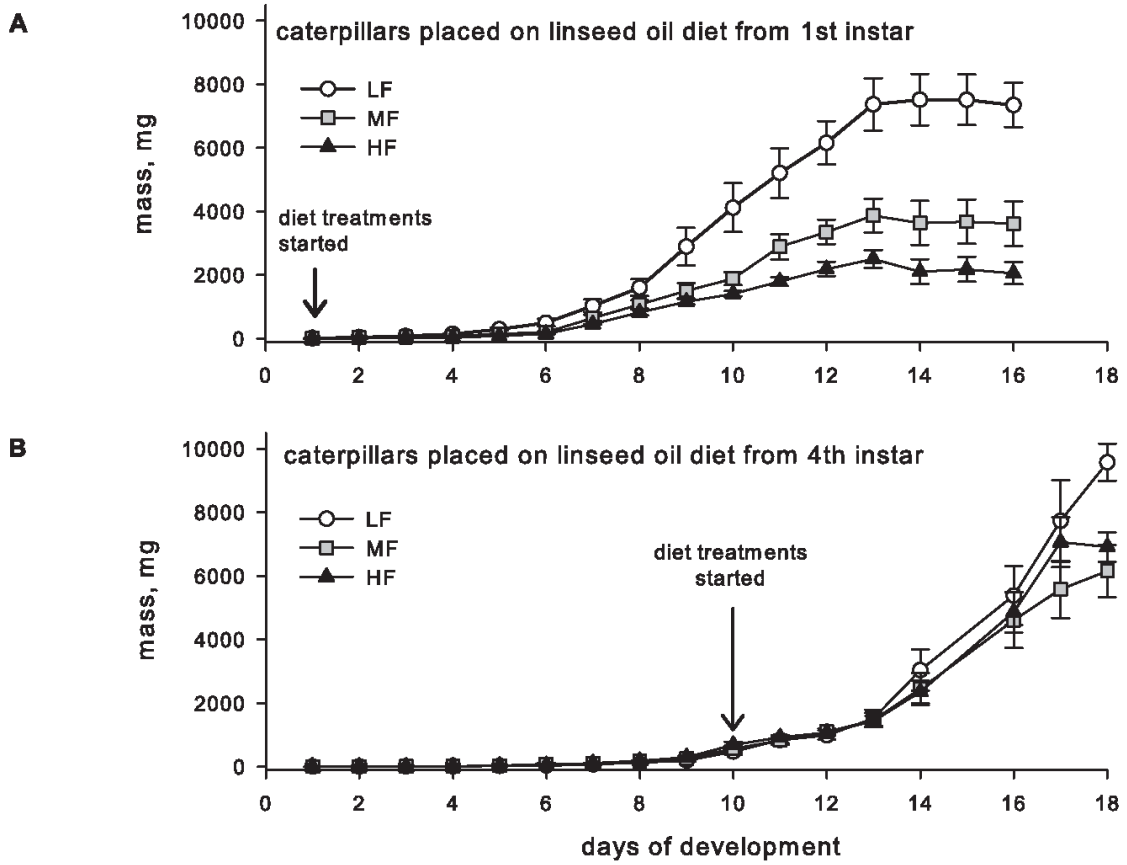

Figure 2. Body masses of caterpillars placed on HF (black triangles), MF (gray squares), or LF (open circles) diets in the first (A) and fourth instars (B). First instar caterpillars were tested on diets made with linseed oil (A). Caterpillars fed HF diets starting in the fourth (B) instar had better survival, but in all cases, HF diets resulted in lower body mass over time.

Table 1. Growth rates of caterpillars on LF, MF, or HF diets.

instar of caterpillar when diet treatment began	Growth rate, (g/day)			F	df	p
	Low-fat	Medium-fat	High-fat			
1	0.46	0.23	0.13	5.4	2, 26	<0.001
2	2.4	1.6	0.87	41	2,21	<0.001
4	9.6	5.7	7.0	5.5	2,26	< 0.025
5	1.7	1.6	1.5	8.1	4,52	<0.001

Because fifth instar larvae accumulate fats in preparation for metamorphosis, we reasoned that their survival should be less impacted by a HF diet, so the rest of the experiments focused on larvae in the fifth instar, and diet treatments were started on day 0 of that instar. Fifth instar caterpillars fed a HF diet in the first experiment had 50% lower body mass compared to caterpillars fed on a LF diet (Fig. 3A; $F_{2,27} = 18.81$, $p < 0.001$; Bonferroni post hoc test, $p<0.03$). In experiment 2 (Fig. 3B), fifth instar mass varied by day ($F_{1,18} = 392.76$, $p < 0.0001$) and by diet fat content ($F_{1,18} = 5.38$, $p < 0.04$). There was no difference between groups on day 0 (1.92 ± 0.09 and 1.77 ± 0.09 for LF and HF respectively). However, by day 4, caterpillars on LF diets were significantly larger than those on a HF diet (9.17 ± 0.48 vs. 7.23 ± 0.48).

In experiment 1, daily growth rates of caterpillars also differed by day depending on the diet they were fed in (Fig. 3C, day x diet interaction, $F_{2,26} = 8.13$, $p < 0.01$). This pattern also held true when looking at mass-specific daily growth rates (data not shown; diet x day interaction: $F_{2, 26} = 3.94$, $p < 0.04$). Caterpillars fed HF diets had daily growth rates that were 6-fold lower and mass-specific daily growth rates that were 80% less than the LF diet-fed caterpillars by day 4 (Fig. 3C; Bonferroni post hoc test, $p < 0.001$). In experiment 2, daily growth rate was significantly affected by day and diet, but not their interaction (Fig. 3D, day: $F_{1,18} = 11.00$, $p < 0.01$, diet: $F_{1,18} = 3.46$, $p < 0.02$), and mass-specific growth rate was only affected by day ($F_{1,18} = 29.33$, $p < 0.001$). Development time was not affected by diet, because the cumulative days to wandering were not significantly different among the three dietary fat levels using either fourth ($F_{2,25} = 1.53$, $p > 0.5$) or fifth ($F_{2,31} = 0.78$, $p > 0.5$) instar caterpillars.

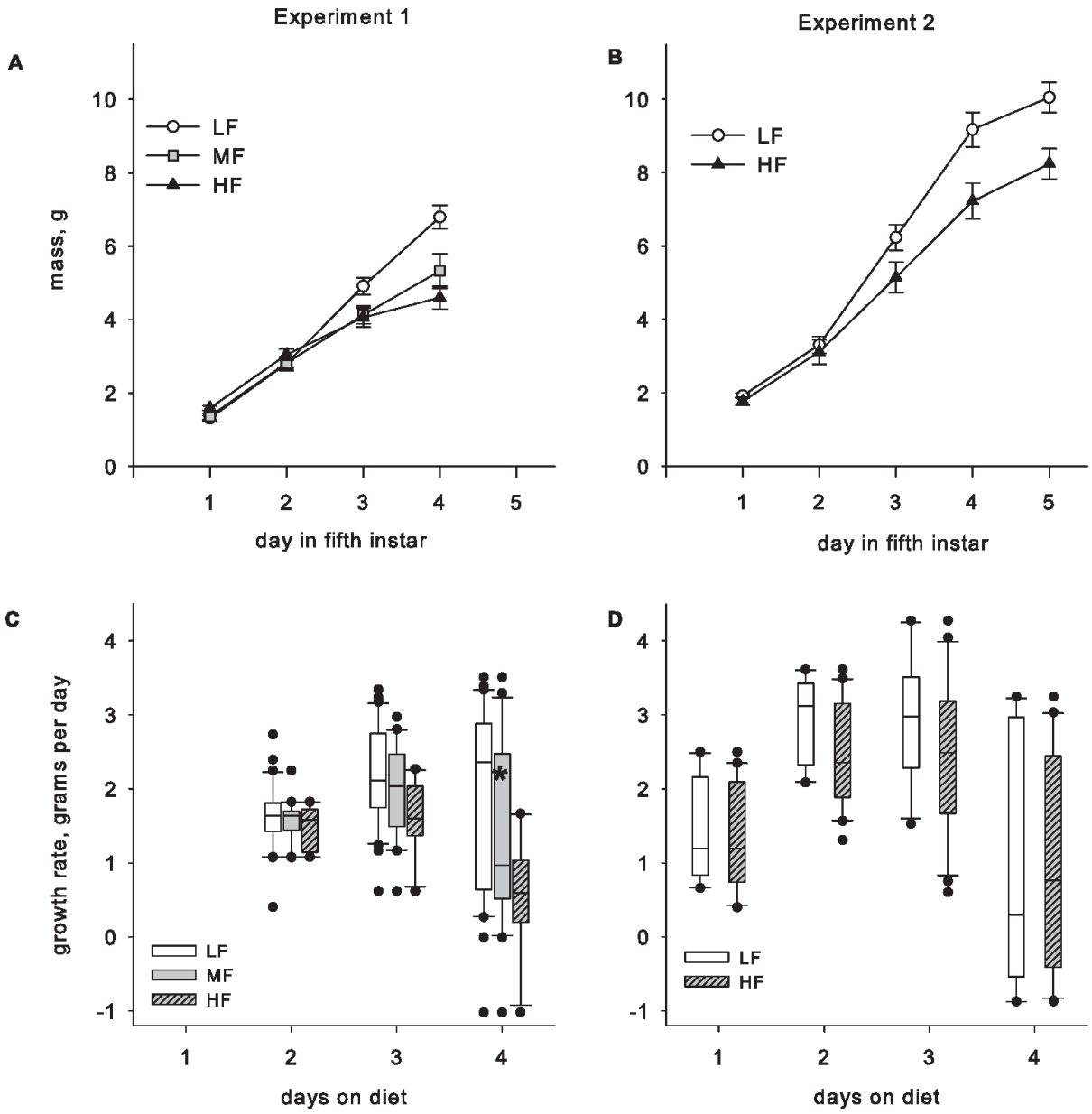

Figure 3. Body mass (A, B) and daily growth rates (C, D) of caterpillars fed HF (black triangles), MF (gray squares), or LF (open circles) diets from the first day of the fifth instar in experiment 1 (A, C) and 2 (B, D). Asterisk indicates a significant difference between growth rates of caterpillars on HF (gray, hatched box) and LF (open box) diets.

Food consumption

Because fifth instar HF diet-fed caterpillars had lower body masses than those reared on the other diets, we tested the hypothesis that it was due to lower food consumption. Food

consumption of fifth instar caterpillars was significantly affected by dietary fat content (Fig. 4A; $F_{2, 27} = 8.32$, $p < 0.01$. By the third day on the diet, HF diet-fed caterpillars consumed a third less of food than LF diet-fed caterpillars (Fig. 4A; Bonferroni post hoc test, $p < 0.03$). Because the HF diet-fed caterpillars were smaller, we also corrected food consumption for body mass. Mass-specific food consumption was not affected by dietary lipid (Fig. 4B; $F_{12,27} = 0.16$, $p > 0.05$), but mass-specific food consumption decreased by day within the fifth instar (Fig. 4B; $F_{1,27} = 11.94$, $p < 0.01$). The correlation between protein consumption and carbohydrate consumption did not vary by diet (Fig. 5A; $F_{1,28} = 0.03$, $p > 0.88$, LF: y= 1.082e-5 + 1.347x; MF: y= -9.159e-16 + 1.347x; HF: y= 3.331e-16 + 1.347x). However, the correlation between protein consumption and lipid consumption varied with diet (Fig. 5B; diet x lipid consumption interaction, $F_{5,24} = 0.03$, $p < 0.0001$, LF: y= 2.228e-6 + 0.2774x; MF: y= -3.261e-16 + 0.4948x; HF: y= -1.388e-17 + 0.7123x).

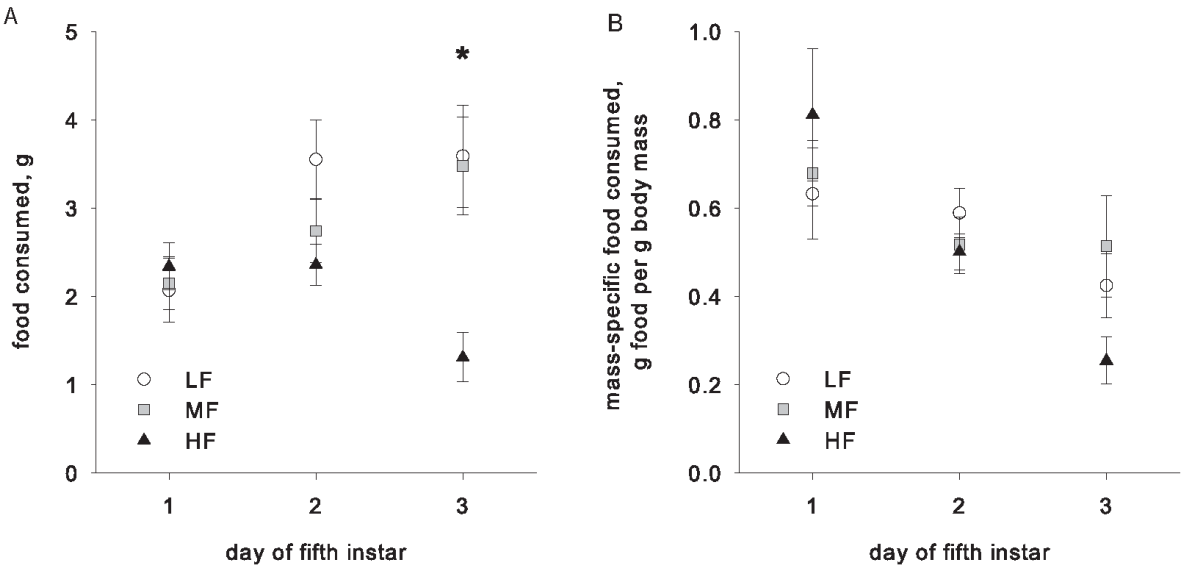

Figure 4. Absolute food consumption (A) and mass-specific food consumption (B) of fifth instar *M. sexta* reared on HF (black triangles), MF (gray squares), or LF (open circles) diets. Asterisk represents significant difference between HF and LF diet-fed caterpillars.

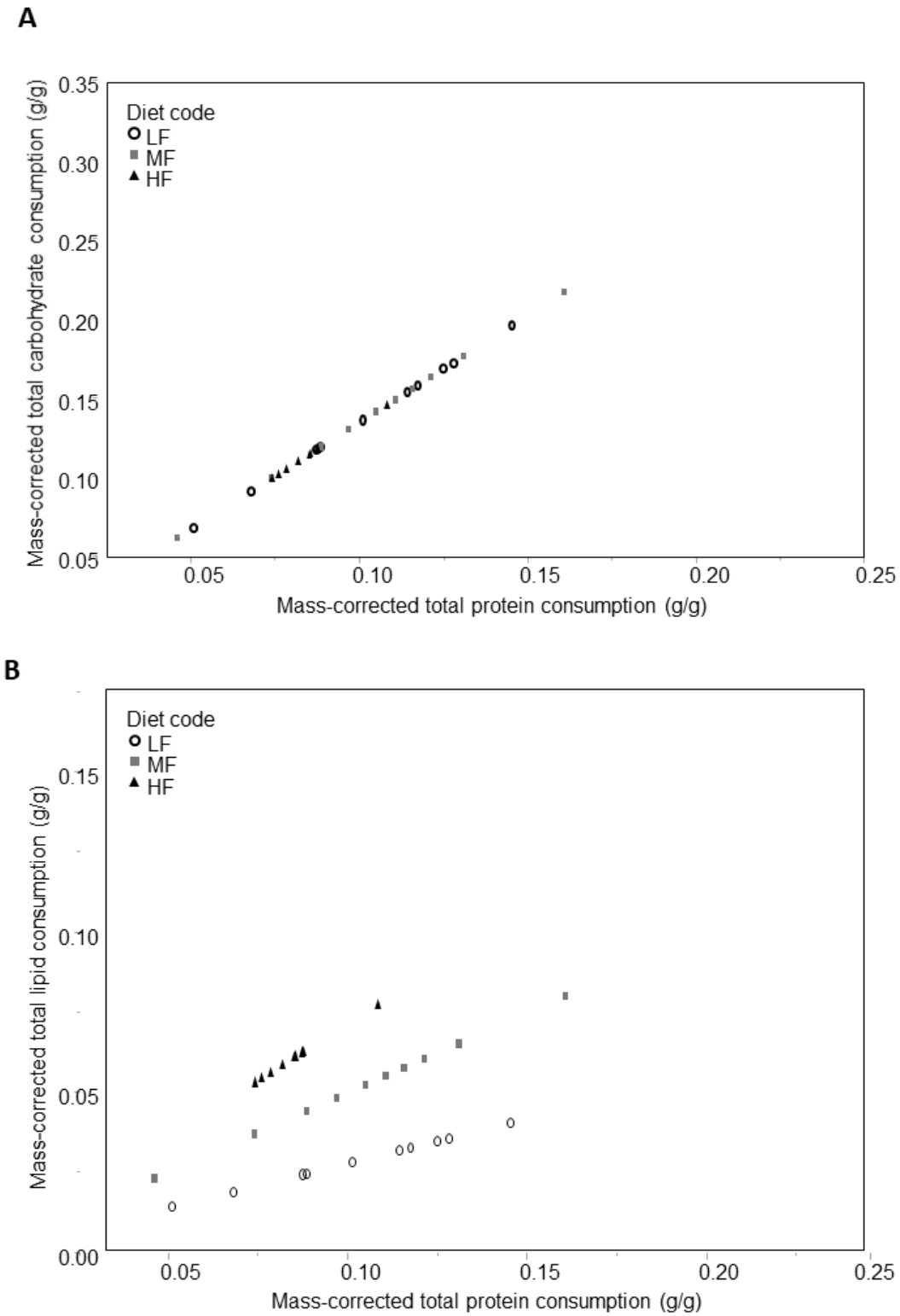

Figure 5. Mass-specific total protein consumption versus (A) mass-specific total carbohydrate consumption and (B) mass-specific total lipid consumption of fifth instar *M. sexta* reared on HF (black triangles), MF (gray squares), or LF (open circles) diets.

Apolipoprotein mRNA expression

Neither absolute (data not shown) or mass-specific APO I and II mRNA expression levels varied with dietary fat content (Fig. 6).

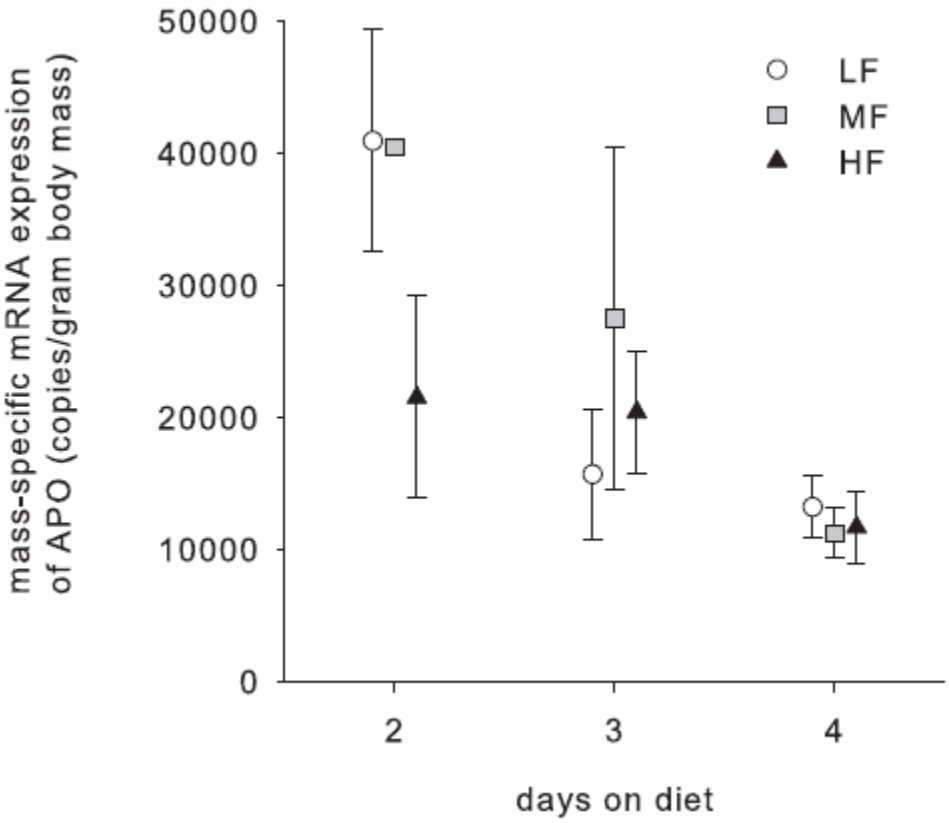

Figure 6. Mass-specific expression of Apolipoprotein I & II mRNA from fat bodies of *M. sexta* on the second, third, and fourth days of the fifth instar after feeding on either HF (black triangles), MF (gray squares), or LF (open circles) diets.

Lipid content of fat body and fecal pellets

Because we found no differences in expression of genes involved in lipid transport, we expected to see changes in either storage or excretion of fat. Neither the wet or dry mass of the fat body varied with dietary fat, although using body size as a covariate in the statistical model showed a significant effect, because caterpillars fed the high fat diet were always smaller (Fig. 7A; wet mass: $F_{1,26} = 70.78$, $p < 0.001$; dry mass: $F_{1,26} = 91.06$, $p < 0.001$). Correcting wet or dry

mass of the fat body for body mass did not reveal an effect of diet nor did it remove the effect of body size (not shown; wet mass: $F_{1,26} = 5.15$, $p < 0.04$; dry mass: $F_{1,26} = 10.8$, $p < 0.01$). Smaller caterpillars had smaller fat bodies and less fat body per gram of body mass. Lipid content of the fat body per mg of either wet or dry fat body mass varied significantly with diet (Fig. 7B; mg lipid/mg wet fat body mass: $\chi 2 = 196$, $p < 0.03$; mg lipid/mg dry fat body mass: $\chi 2 = 193$, $p < 0.04$). HF diet-fed caterpillars stored more than double the amount fat per mg of fat body compared to LF-fed caterpillars.

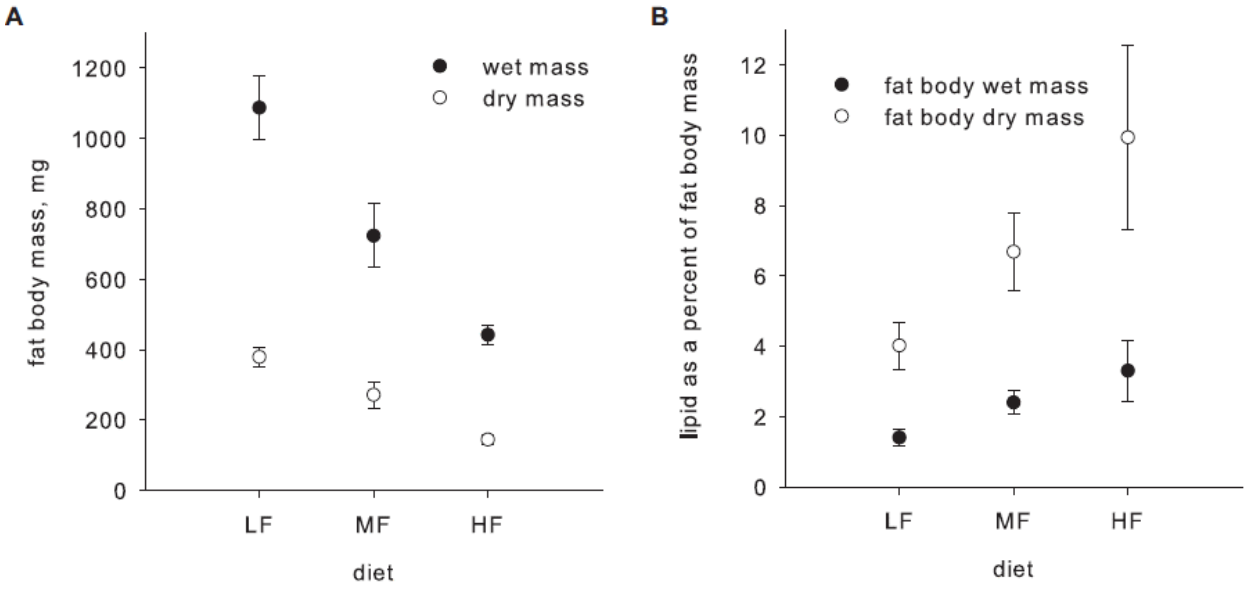

Figure 7. Effects of dietary fat content on wet and dry mass of fifth instar *M. sexta* fat body mass (A) and lipid content relative to fat body mass (B).

For caterpillars fed LF diets, the number of fecal pellets collected increased throughout the instar, but those fed HF diets excreted fewer pellets as they aged, regardless of experiment (not shown; experiment 1: day x diet interaction, $F_{1,27} = 6.35$, $p < 0.006$; experiment 2: effect of day $F_{1,18} = 14.45$, $p < 0.01$ and effect of diet, $F_{1,18} = 22.86$, $p < 0.001$). Wet masses of single fecal pellets varied differently with diet depending on the age of the caterpillar in experiment 1 (Supplemental Fig. 3; day x diet interaction, $F_{2,27} = 15.88$, $p < 0.001$), but in experiment 2 there

was no effect of diet (Supplemental Fig. 3; effect of day, $F_{1,15} = 56.28$, $p < 0.001$). When we corrected both fecal pellet number and mass for body mass, the effect of diet disappeared ($p > 0.05$ for number of pellets, wet mass, and dry mass of fecal pellets).

The amount of lipid excreted in the fecal pellets varied differently by day depending on which diet caterpillars were fed (Fig. 8A; day x diet $F_{2,11} = 4.47$, $p < 0.04$). HF diet-fed caterpillars excreted nearly 50% more lipid than LF diet-fed caterpillars on the fourth day of fifth instar (Fig. 8A; Bonferroni post hoc test, $p < 0.03$). Lipid content per mg of fecal pellet dry mass was significantly affected by body mass on day 3 (Fig. 8B; $F_{1,11} = 8.31$, $p < 0.02$) and by an interaction between day and diet ($F_{4,22} = 3.25$, $p < 0.04$). When we corrected the lipid content of the fecal pellet by body mass, we found a significant interaction between dietary fat content the numbers of days they had been fed on a given diet (Fig. 8C; $F_{2,12} = 5.34$, $p < 0.03$).

Approximate digestibility

Approximate digestibility (AD) of all diets increased with the caterpillars' age within the fifth instar (Fig. 9; effect of day: $F_{1,25} = 4.42$, $p < 0.05$) and with dietary fat content (effect of diet: $F_{2,25} = 8.21$, $p < 0.01$). AD for the HF diet was about 10% higher than for the MF or LF diet, until day three. On day three, AD of the LF diet increased to a level comparable to the HF diet (Fig. 9; Bonferroni post hoc test, $p < 0.03$).

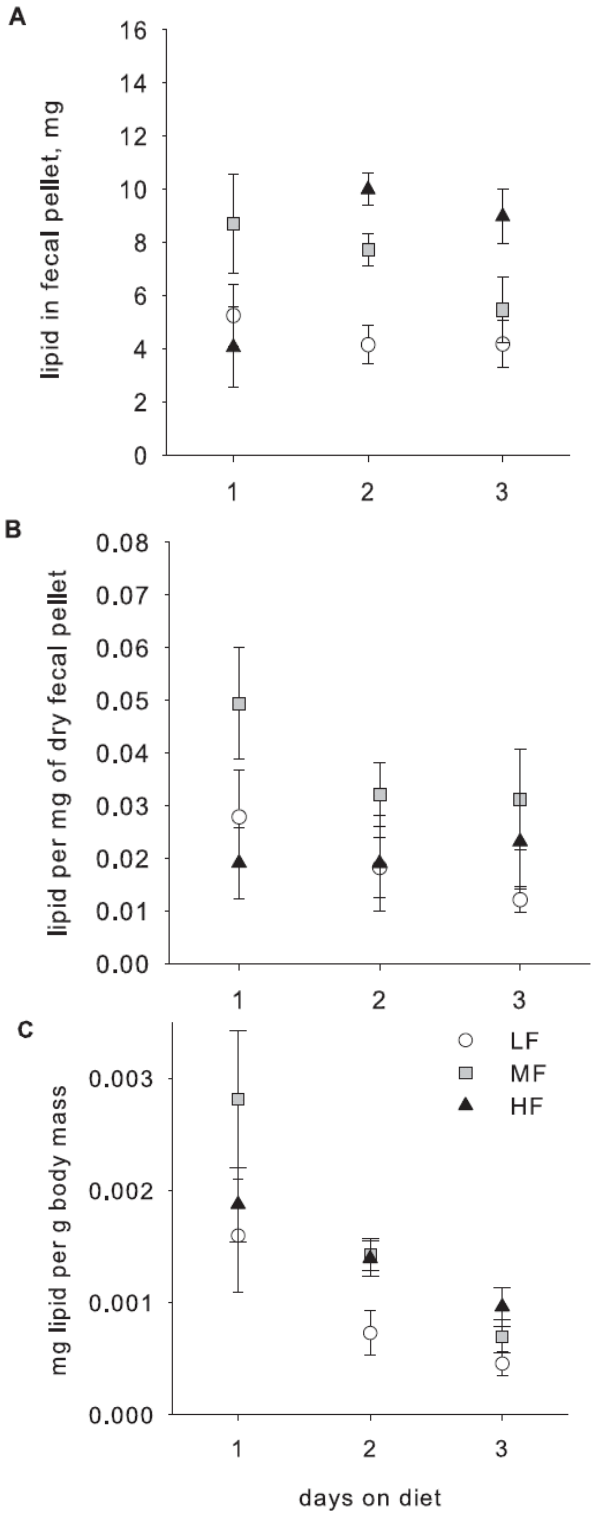

Figure 8. Lipid content of fecal pellets of fifth instar *M. sexta* fed HF (black triangles), MF (gray squares), and LF (open circles) diets (A), relative to the dry mass of fecal pellets (B), and relative to body mass (C)

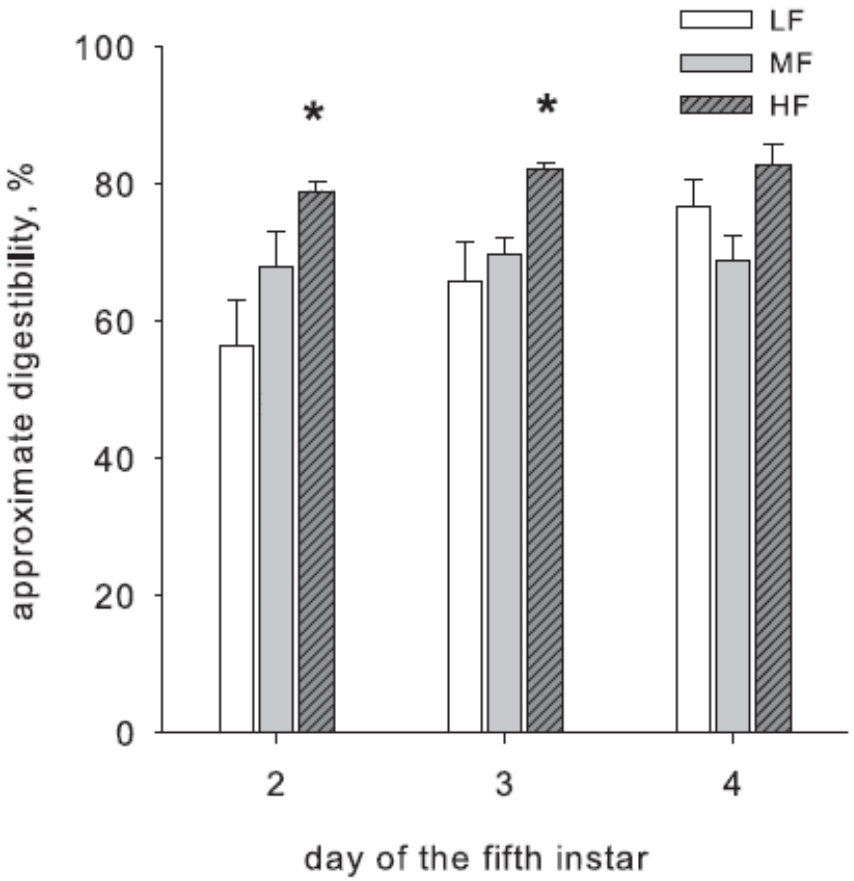

Figure 9. Approximate digestibility of each diet by day of the fifth instar in *M. sexta*. Asterisks indicate a significant difference between HF (gray, hatches box) and LF (open box) diets.

Discussion

This study focused on the impacts of dietary fat content on development and performance and is the only study to show high-fat diet-induced anorexia in an insect, contradicting the current knowledge on the effects of high-fat diets. Caterpillars reared on a high-fat diet were smaller and had decreased growth rates. This likely resulted from a reduction in consumption, as HF-fed caterpillars consumed one third less than caterpillars fed diets with less fat. The decrease in consumption we measured directly was corroborated by the decrease in fecal pellets from those caterpillars. However, the lipid contents of fat bodies and fecal pellets varied by day

throughout the fifth instar, showing a change in lipid retention with development. We initially thought the increased lipid content impacted food digestibility and consumption. However, approximate digestibility was higher for the high-fat diet, indicating that digestibility was not a factor. There were also no changes observed in ApoI or ApoII, suggesting that alterations in lipid transport proteins were also not a cause of decreased lipid storage. Together, these data suggest that high-fat diet-fed caterpillars consumed food until reaching a maximum lipid storage capacity. Additionally, we looked at total lipid intake to see if that varied as a possible explanation. Total lipid intake for low-fat diet-fed caterpillars was less than both the medium and high-fat diet groups. Since there was no difference between the medium and high-fat diet-fed caterpillars, we hypothesize a lipid intake threshold may exist between the low and medium dietary fat levels. Once that limit is reached, we propose that a feedback mechanism may disrupt feeding, at which time caterpillars switch from lipid storage to excretion, resulting in reduction of feeding.

Along with our proposed feedback mechanism, our results raise new questions when compared to the literature. Previous studies that altered dietary fat content showed no effect on caterpillar size, suggesting that feeding was not altered those studies (Fernando-Warnakulasuriya et al., 1988; Tsuchida and Wells, 1988). It is unclear why our results differed, especially since the dietary fat levels we chose were based on those studies. This study produced the same results when repeated several times, suggesting that something between our repeated study and other studies differed. A possible difference between these studies could be the variation in fatty acid composition between soybean oil (Fernando-Warnakulasuriya et al., 1988) and the linseed oil used in our study. It is also possible that commercially available *M. sexta* have varied genetically

or adapted to living on an artificial diet over time, explaining the difference between previous studies and ours (D'Amico et al., 2001; Kingsolver, 2007; Kingsolver et al., 2009).

It is unclear if time and mass rearing of *M. sexta* on an artificial diet may have altered lipid metabolism mechanisms. Herbivorous insects like *M. sexta*, ingest lipid in the form of phospholipids and triacylglycerols, both of which must be digested before being used (Beenakkers et al., 1985). Triacylglycerols are hydrolyzed into free fatty acids and glycerol, which are absorbed by the walls of the midgut lumen (Canavoso et al., 2001; Turunen and Chippendale, 1977), and transported by lipoproteins through the hemolymph as diacylglycerol for use or storage (Arrese et al., 2010; Canavoso et al., 2001). In insects, lipoprotein acts as a reusable shuttle for lipid transport and can transport varying types of lipid straight to specific tissues (Canavoso et al., 2001; Ryan et al., 1990). Although they were smaller, HF-diet caterpillars had higher lipid content in their primary storage organ, the fat body. Similar to our results, another study using fifth instar *M. sexta* showed that a high-fat diet caused higher deposition of fat in the fat body and increased diacylglycerol content of lipophorin extracted from hemolymph (Fernando-Warnakulasuriya et al., 1988). Although we did not measure the lipid content of hemolymph or of lipophorin specifically, we would expect that these would have been higher similar to fat body storage. In preliminary data, Fernando-Warnakulasuriya et al. (1988) found that when reared on a natural leaf diet, *M. sexta* consumed lower levels of lipid (approximately 0.14%), but lipid content of their isolated lipophorin was similar to that of larvae on laboratory diets. Our results are consistent with this and previous work showing that lipophorin synthesis does not vary with the amount of lipid in the diet (Ryan et al., 1986a; Ryan et al., 1986b). According to this same study, a natural leaf diet (0.14%) has a lower proportion of fat than the usual laboratory diet (1.2%) (Fernando-Warnakulasuriya et al., 1988). These

numbers are based on tobacco plants, but *M. sexta* can also consume *D. innoxia*, which can have as much as 7% lipid in its leaves. Although both plants are from the same order, Solanales, the lipid composition of their leaves seem to be drastically different. One can conclude that lipid storage for wild *M. sexta* would be highly variable depending on host plant availability and would be overall drastically different than lab-reared larvae. Therefore, it is difficult to discuss the ecological relevance of the lipid content used in this study.

The additional lipid content did increase the overall caloric value of each diet, making the HF diet not isocaloric. Therefore, the resulting decrease in feeding could have been due to the associated high calories. The caloric difference per diet was an additional 105.5 and 211 kilocalories per liter of the medium and high-fat diets, respectively. Effects of caloric intake have been studied in other insects, such as the fruit fly *Drosophila melanogaster*. Diet restriction has been argued to mediate life span in adult *D. melanogaster* by limiting specific nutrients instead of calories (Mair et al., 2005). However, caloric intake was not directly measured, leading others to argue the effect of caloric intake to not be dismissed. Min et al. (2007) repeated this study to test whether the energetic value of diets was proportional to caloric intake and found that flies reared on an isocaloric diets did differ in caloric intake and lifespan. They also found that flies fed a high yeast/low sugar diet gained more weight, regardless of their diet having the same caloric value as other diets (Min et al., 2007). Our results were similar with low-fat diet-fed caterpillars being the largest in mass. However, when adjusted for total food intake, all groups consumed on average the same number of total calories. This suggests that the HF diet caused a decrease in feeding not because of the increased calories but because of the increased fat content. However, additional studies would be needed to separate the effects of caloric content and fat

content on feeding. Although studied in other insect species, our understanding of the effect of caloric intake on insect development and nutrient balance still seems to be unclear.

Traditionally, dietary self-selection is thought to be the mechanism for balancing nutrients. When pre-conditioned to a diet lacking a specific nutrient, insects will consume more of a diet to obtain enough of the missing nutrients (Mayntz et al., 2005; Ojeda-Avila et al., 2003; Rowell and Simpson, 1992; Thompson and Redak, 2000, 2005; Thompson et al., 2001). The nutritional rule of compromise explains that insects eating an imbalanced diet will compromise between underingesting some nutrients at the cost of overingesting others (Raubenheimer, 2011; Raubenheimer and Simpson, 1997). When analyzing mass-corrected total protein consumption versus non-protein consumption, our results showed no effect of diet in carbohydrate and protein intake, which was expected since those nutrients were held constant across diets. We did see a negative correlation between lipid and protein intake that varied by diet. This suggests that there was a compromise between overingesting lipid and underingesting protein, preventing larvae from reaching their target protein intake. However, additional studies would be needed to truly test how lipid intake varies with other nutrients.

Studies in both predatory and non-predatory insects demonstrate that selective feeding can change in response to nutritional requirements for development and reproduction (Barton Browne, 1995; Bowen, 1992; Browne and Raubenheimer, 2003; Jensen et al., 2012; Jones and Raubenheimer, 2001; Mitchell and Briegel, 1989; Noreika et al., 2016; Raubenheimer, 1992; Raubenheimer and Browne, 2000; Raubenheimer and Jones, 2006; Raubenheimer et al., 2007; Robich and Denlinger, 2005; Socha et al., 1998; Stockhoff, 1993; Zhou et al., 1995). Changes in diet preferences are also induced by other factors like hemolymph trehalose levels and sickness (Adamo, 2005; Nirupama, 2015; Thompson and Redak, 2000). Waldbauer and Friedman (1991)

introduced the “malaise hypothesis” which stated that insects will eat a diet deficient of a major nutrient until it causes a metabolic stress. This stress acts as signal and stimulates the insect to search for a food that fulfills the nutrient requirement (Adamo et al., 2010; Adamo et al., 2007; Friedman et al., 1991). Therefore, high-fat diet-fed larvae may have felt “sick” from consuming large amounts of fat, resulting in a sickness-induced anorexia phenotype. Alternatively, it is possible that a nutrient threshold for lipid storage is causing a feedback for caterpillars to eat less of it, resulting in smaller, more lipid-concentrated caterpillars.

Although caterpillars fed HF diets stored significantly more lipid in the fat body, they also excreted more lipids, supporting the hypothesis that there may be an upper limit of internal lipid that caterpillars will tolerate. There is no other evidence of a lipid threshold, but thresholds have been shown for other dietary components. *M. sexta* larvae have shown to excrete excess dietary phosphorous with little retention in the body (Woods et al., 2002), resulting in lower growth rates (Perkins et al., 2004). Zehnder and Hunter (2009) investigated the effects of excess nutrient levels on herbivorous insect growth, with their results showing a negative effect of high nutrient concentrations on aphid development. Their data along with other similar studies (Raubenheimer and Simpson, 2004), support a threshold elemental ratio hypothesis (Boersma and Elser, 2006). This hypothesis states that excreting excess nutrients can be energetically costly, making consumption of a lower quality food or high combination of nutrients optimal for growth and development, versus high concentrations of individual nutrients. Interestingly, HF diet-fed caterpillars began excreting excess lipid rather than showing compensatory eating until reaching a critical weight. This supports the hypothesis and matches previous research showing that insects fed an imbalanced diet will store the excess of unwanted nutrients (Raubenheimer, 1992; Raubenheimer et al., 2007). Within 48 hours after self-selecting a HF diet, predatory

beetles *Agnom dorsale* had an increase in lipid storage from 14% to 46% by dry mass (Raubenheimer et al., 2007). To test the hypothesis that there is an upper threshold for lipid, we calculated total lipid consumed and used an ANOVA to determine whether the effect of diet. Total lipid intake varied ($p < .0001$), however, unlike the rest of our results, it only differed between the LF and MF groups. The lack of difference between the MF and HF diets suggest that an upper threshold may exist somewhere between these two lipid levels as a result of balancing lipid intake, storage, and excretion.

In addition to increasing their lipid reserves to prepare for pupation and reproduction, 5th instar *M. sexta* must first continue larval development to induce commitment to metamorphosis. Achieving a critical weight (CW) is known to be an important factor for metamorphosis induction (Davidowitz et al., 2003; Helm et al., 2017; Nijhout et al., 2006; Nijhout et al., 2014; Shingleton, 2011). In our study, larvae reared on high-fat diets were able to continue development despite not reaching a critical weight, suggesting an alternative mechanism is at hand. We hypothesize that a nutritional threshold, especially for a vital nutrient like lipids, may be inducing larvae to commit to metamorphosis independently of body size since HF diet-fed larvae were all smaller. In support of commitment independently of body size, Helm and Davidowitz (2015) showed that transplanting hemolymph of post-critical weight *M. sexta* into caterpillars that had not yet reached critical weight shortened their growth duration. Similarly, in the solitary bee, *Osmia lignaria*, food absence can also induce metamorphosis commitment rather than bees reaching a critical weight (Helm et al., 2017). During normal development, changes in hormone levels cause larvae to stop feeding and prepare for pupation (Nijhout and Williams, 1974). These studies suggest that altering components in circulating hemolymph either directly through transplant or indirectly through diet manipulation, can induce commitment to

metamorphosis without reaching a critical weight. Whether these cues act directly, or indirectly by altering nutrient sensing pathways, is unclear.

Insect body size has been shown to be determined by pathways like the insulin/insulin-like growth factor signaling (IIS) pathway and nutrient sensing Target of rapamycin (TOR) pathways (Hatem et al., 2015; Koyama and Mirth, 2018; Mirth et al., 2014; Smith et al., 2014; Stern, 2003). It is possible that HF diet or the components of the lipid itself may act as stimulant signaling the brain and the IIS/TOR pathways to inhibit feeding once a threshold level of the nutrient is reached. An additional study adding endogenous lipid in attempt to induce metamorphosis would verify if lipid thresholds can indeed be important for development. Although our studies were terminated at the wandering stage prior to metamorphosis, anecdotal evidence from a few moths that were reared to adulthood showed that the moths from MF-fed caterpillars had more eggs than those reared on HF or LF diets (unpublished data). As different developmental stages have different requirements, it makes sense for insects to change their dietary preference to meet those demands.

Conclusion

Nutritionally balanced diets are crucial, but the required balance can vary depending on development, sex, species, and other physiological factors. Although our experiments were conducted in a controlled setting, the reality is that insects reared in the lab may not be indicative of what is happening in nature. Future studies comparing multiple host plants and artificial diets would be essential to better understand these differences. Insects are naturally exposed to many factors such as predation, changes in climate, and pathogens, which can all alter nutrition. How all of these impact nutrition and lipid metabolism is still to be answered.

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CHAPTER 3. TOLL-MEDIATED SUPPRESSION OF INSULIN SIGNALING IN RESPONSE TO SICKNESS-INDUCED ANOREXIA IN THE HORNWORM, *MANDUCA SEXTA*

Abstract

Both vertebrates and invertebrates exhibit sickness-induced anorexia (SIA), an immune response that causes cessation of feeding during an immune challenge. The mechanism underlying SIA that ties feeding to immunity has not yet been identified. In vertebrates, insulin signaling regulates many physiological processes, including feeding. Because of its role in regulating feeding, I hypothesized that the insulin signaling pathway also plays a role in SIA in insects. I used a well-established insect model of immunity, the tobacco hornworm, *Manduca sexta*, to test this hypothesis. First, I investigated the effects of insulin signaling on growth and development during SIA. I manipulated insulin signaling in caterpillars that were injected with vehicle or *E. coli* to exhibit SIA. Manipulating insulin signaling had no effect on larval growth with no difference between treatment groups in body mass or feeding. However, adding extra insulin during SIA delayed adult emergence by 3 days. To determine what genes may be involved in the SIA response, I measured expression of target genes of the insulin signaling pathway and intracellular signaling pathways, such as Toll, MAPK, and JAK-STAT. Since insulin-producing cells in the brain are regulated by signals from the fat body and are the primary site for ILP secretion, I measured expression levels of genes from both fat body and brain samples. For both tissues, the majority of the differences were between the SIA and other treatment groups, similar to results seen in the first experiment. Target genes of the Toll pathway were all upregulated, indicating the pathway as a whole was upregulated. Changes in gene expression in both JAK-STAT and MAPK-JNK-p38 pathways suggested neither were activated.

Several genes in the insulin signaling pathway were upregulated. Interestingly, FOXO was also upregulated despite its inhibitor also being upregulated, indicating another mechanism is regulating FOXO. I also saw upregulation of the Toll pathway which has been shown to suppress the insulin signaling pathway to allow for FOXO expression. Previous evidence showed that the Toll pathway suppresses insulin signaling during an immune challenge to enhance immune responses. Suppressed insulin signaling leads to elevated levels of FOXO mRNA, which contributes to increased stress tolerance. My findings support these mechanisms with both FOXO and Toll pathway genes being upregulated, despite upstream inhibitors of FOXO also being upregulated. Therefore I propose a Toll-mediated suppression of the IIS pathway as a potential mechanism behind SIA in *M. sexta*.

Keywords: insect, illness-induced anorexia, wortmannin, development, Toll signaling

Introduction

Most animals stop feeding when their bodies become infected with pathogens; a condition called sickness-induced anorexia (SIA). Most animals that have been studied exhibit this phenomenon, yet its underlying mechanism and the adaptive significance of SIA are still not understood. Understanding why animals stop feeding when ill is of practical importance, especially for humans, because not feeding and hence altering nutritional status can be detrimental. In humans, metabolic diseases such as diabetes mellitus are drastically impacted by changes in nutrition and make individuals highly susceptible to infections, making periods of non-feeding like SIA potentially harmful (Shilling and Raphael, 2008). In tobacco hornworms, SIA causes decreased growth and delayed development (Booth et al., 2015). Although the period of non-feeding during SIA could be beneficial at first, not feeding could also become harmful to the organism over time (Adamo et al., 2010; Langhans, 2007).

SIA is hypothesized to be an adaptive function that causes an energy reallocation towards immune function (Adamo, 2005; Adamo et al., 2010; Adamo et al., 2007; Mason et al., 2014; Povey et al., 2014; Singer et al., 2014). Energy reallocation due to SIA could enhance survival by ensuring that the immune system has enough resources to resist or fight infection. Even with clinical and experimental evidence of SIA, the underlying function and mechanism behind SIA and its role in immunity remains unresolved (Adamo et al., 2010; Adamo et al., 2007; Bluthe et al., 2006; Dantzer and Swanson, 2012; Dantzer, 2001a, b, 2004; Dantzer and Kelley, 2007; Faggioni et al., 1997; Kelley et al., 2003; PlataSalaman, 1996, 1997; Povey et al., 2014). Because SIA results from changes in both nutritional signaling and the immune system networks, it is important to find where these systems connect.

One possible connection in insects is the insulin/insulin-like growth factor signaling (IIS) pathway. Both immune-related and IIS pathways are integrated at the fat body, the primary site for protein synthesis, including anti-microbial peptides (AMP; Arrese and Soulages, 2009). In addition to being the primary site for immune system signaling, the fat body plays a key role in assessing nutritional status, via trehalose concentration and amino acid content in the hemolymph, defined as nutritional signaling (Anand and Lorenz, 2008; Arrese et al., 2010; Arrese et al., 2008; Azeez et al., 2014). The fat body is also the site of expression of genes in the insulin/insulin-like growth factor signaling (IIS) pathway, which regulates nutrient stores (Geminard et al., 2009; Nassel and Vanden Broeck, 2016).

In animals across a wide range of taxa, the IIS pathway plays a role in regulating development, growth, metabolism, and reproduction (Bates et al., 2013; Cheng et al., 2005; Das and Arur, 2017; Das and Dobens, 2015; Dupont and Holzenberger, 2003; Dupont and Scaramuzzi, 2016; Kuczkowski and Brinkkoetter, 2017; Nässel et al., 2013; Nijhout et al., 2014;

Tatar et al., 2003). Hormonal peptides with highly conserved sequence and structure to insulin, insulin-like peptides (ILP) have been found in insects, but their physiological functions are still being revealed (Aslam et al., 2011; Mirth et al., 2014; Mizoguchi and Okamoto, 2013; Nassel, 2012; Nässel et al., 2013; Nassel and Vanden Broeck, 2016; Wu and Brown, 2006). Functions of bombyxin-II, an ILP in *Bombyx mori*, include blocking synthesis of blood sugar (trehalose) from dietary sugars and amino acids, which indirectly lowers blood sugar, much like mammalian insulin (Satake et al., 1997). One of the known functions of ILP in *Drosophila* is to mobilize energy stores to enhance immune responses during infection or even starvation (Dionne et al., 2006; Libert et al., 2008). However, during starvation IIS was decreased, contradictory to results shown in B. mori (Liu et al., 2010). The decrease in IIS released inhibition of the IIS pathway downstream target FOXO, which resulted in production of antimicrobial peptides (AMP) (Becker et al., 2010). Genes in the IIS pathway are highly expressed in fat body tissues of the silkworm, B. mori, and are upregulated during molting and pupation, which are non-feeding periods (Liu et al., 2010), suggesting that expression of the IIS pathway may be involved in regulating feeding in insects. In *B. mori*, starvation resulted in elevated expression levels of several targets in the IIS pathway in the fat body (Liu et al., 2010). With insulin signaling changing in response to starvation and leading to AMP production, these results suggest a role for IIS in stress responses and innate immunity in insects.

The focus of this study was to determine the role of insulin signaling in SIA. I used the tobacco hornworm, *Manduca sexta*, because they have been shown to exhibit SIA, and the link between IIS and immune functions has been elucidated in this system. I hypothesized that insulin signaling regulates cessation of feeding in *M. sexta*. To test this hypothesis, I manipulated the insulin signaling pathway and measured the impact on growth and development during SIA and

in control 5th instar *M. sexta*. To determine how insulin signaling changes during SIA, I measured expression of target genes of the insulin signaling and associated intracellular signaling pathways such as Toll, MAPK, and JAK-STAT in fat body and brain tissues. Because the fat body plays a role in relaying nutritional information and processing information from the immune system, it is very likely to be involved in SIA.

Materials and methods

Experimental design

To test the effects of insulin signaling on SIA, a 2x3 factorial experiment was conducted on 5th instar day 1 larvae with SIA treatment groups (PBS/*E. coli*) and insulin manipulation treatment groups (PBS/Wort/Insulin). SIA treatment groups consisted of a vehicle-injected group (PBS, phosphate buffered saline), and an SIA group (*E. coli*) that was injected with heat-killed DH5-α *E. coli*, a laboratory-made bacterial strain. The insulin manipulation treatment groups consisted of a vehicle-injected group (PBS), a "blocked insulin" group that was injected with a downstream insulin signaling inhibitor (wortmannin), and an extra insulin group that was injected with bovine insulin.

To determine which genes may be involved in SIA, I measured expression of genes in the insulin signaling pathway and immunity-related genes, using 2x3 factorial experiment, with SIA treatment groups (Control/PBS/*E.coli*) in two tissue types. Since ILP are secreted from both fat body and insulin-producing cells in the brain of M. sexta, those two tissues were used. SIA treatment groups consisted of non-injected, handled only (Control), sham-injected (PBS), and bacteria-injected (SIA).

Animal rearing

Manduca sexta larvae were purchased from Carolina Biological Supply (Burlington, NC) and reared at 25°C with a photoperiod of 16L:8D on a wheat-germ-based artificial diet as previously described (Vishnuvardhan et al., 2013). Larval age was tracked by observing the presence of a head capsule, indicating larvae were ready to molt and progress to the next developmental stage (Lundquist et al., 2018).

Bacterial cultures

E. coli cultures were prepared overnight to reach stationary phase and concentrated to a working concentration of 1 x 10^6 CFU/10µl. Bacteria in solution were heat-killed by placing in a 65˚C water bath for 30 min.

Drug preparations

Phosphate buffered saline (PBS) was used as a vehicle control for SIA. Bovine insulin (I4011 SIGMA, 50mg) was dissolved in 10 mM HCl (Koyama et al., 2008). Stock solution was diluted to working concentrations of 0.1, 1, 10, and 100M. Wortmannin (BML-ST415-0001 ENZO) was dissolved in 10% DMSO (Okada et al., 1994) and diluted to working concentrations of 0.01, 0.1, and 1µM. 10% DMSO, or 10mM HCl were used as vehicle controls in preliminary studies.

Injections

Larvae were anesthetized on ice for 10 min while treatment injections were prepared. A 10-µL syringe (Hamilton, Reno, NV) was sterilized with 75% ethanol, placed in a micro-manipulator (Prior Scientific Instruments, Cambridge, UK) and fitted with a sterile, 26-gauge needle (EXELINT, Redondo Beach, CA). The injection site, between the first and second proleg and the 3rd and 4th spiracle, was cleaned with ethanol before injection. Holding the lower

abdomen, the needle tip was placed on the surface of the skin and slowly inserted. Upon correct needle insertion, 10µl was slowly injected and the needle removed. The injection sites were re-sterilized before returning larvae to their cups. Because larvae were to receive two injections, one for each treatment, I conducted preliminary studies to determine the appropriate timing of the second injection. There was no effect of injection time (from 6 h to 24 h) on body mass, feeding, or developmental timing within treatment groups (Fig. B1), so I used 6h post-first injection for second injections.

Sickness-induced anorexia

The phenotype of SIA, cessation of feeding, was assessed by weighing caterpillar food before it was placed in cups and daily thereafter. Additional cups containing pieces of food of the same size were used to determine the rate of water loss of 0.26g/day. In addition, caterpillars were weighed daily to calculate growth rates. Caterpillar housing contained a platform made of hardware cloth so that fecal pellets would drop below for daily collection. Fecal pellets were counted and weighed daily to validate feeding data.

NanoString nCounter

M. sexta were reared as described above, and upon day 1 of the fifth instar they were separated into treatment groups. Treatment groups consisted of non-injected (control), sham-injected (vehicle control), and bacteria-injected (SIA). Tissue samples were collected at 8 hours post-injection since immune responses peak after 8 hours. Larvae were anesthetized by submersion in ice for 10 minutes, then placed on a dissection plate and covered in a saline buffer. Fat body (n=6/group) and brain tissue (n=3/group) samples from each treatment group were dissected, and RNA was extracted with TRIzol (Invitrogen, Life Technologies, Grand Island, NY, USA) according to manufacturer's instructions. Due to the size of the brain tissue yielding

low RNA quantities, each brain sample consisted of two pooled samples. RNA pellets were diluted to a target concentration of 20 - 50 ng/µL, loaded into a 96-well skirted plate, and stored in -80˚C. A custom probe codeset for *M. sexta* was designed and manufactured for 30 genes of interest in the insulin, TOLL, and JAK-STAT pathways (NanoString Technologies Inc., Seattle, WA, USA) (Table C1). Plates were shipped on dry ice to the University of Minnesota Genomics Center (Minneapolis, MN) for processing with nCounter® Analysis System (NanoString Technologies Inc.). Data were normalized to the geometric mean of 5 reference genes.

Statistical analysis

IBM SPSS (v. 23 SPSS Inc. 2016) and JMP PRO (v.14.0, SAS Institute Inc., 2018, Cary, NC, USA) software were used for statistical analysis. Data were tested for normal distribution and for homogeneity of variances using Levene's test. Differences among treatment and control groups in body mass, growth rate, food consumption, and other measurements over time were analyzed by repeated measures analysis of variance (RM-ANOVA). Because gene expression data were not normally distributed, nCounter data were analyzed with a Kruskal-Wallis test for differences between treatment groups by tissue sample. Bonferroni-corrected post hoc tests were used to determine which groups differ. P-values less than 0.05 indicate significant differences among means. Results are shown as means ± standard error throughout.

Results

Effects of insulin signaling on growth and development during SIA

To test whether insulin signaling plays a role in SIA, I manipulated insulin signaling (PBS/Wort/Insulin) in caterpillars that were either vehicle-injected (PBS) or injected with E. coli to exhibit SIA. Manipulating insulin signaling had no effect on SIA, because larval growth did not differ between treatment groups when looking at body mass (Fig. 10A). Feeding was also

only affected by SIA ($F_{1,35}$ = 13.787, p < 0.001), but not insulin manipulation (Fig. 10B). However, increasing insulin during SIA as a larva had long term effects, delaying adult emergence by 3 days (Fig. 10C, insulin x immune challenge interaction: $F_{2,34}$= 12.647, p < 0.001).

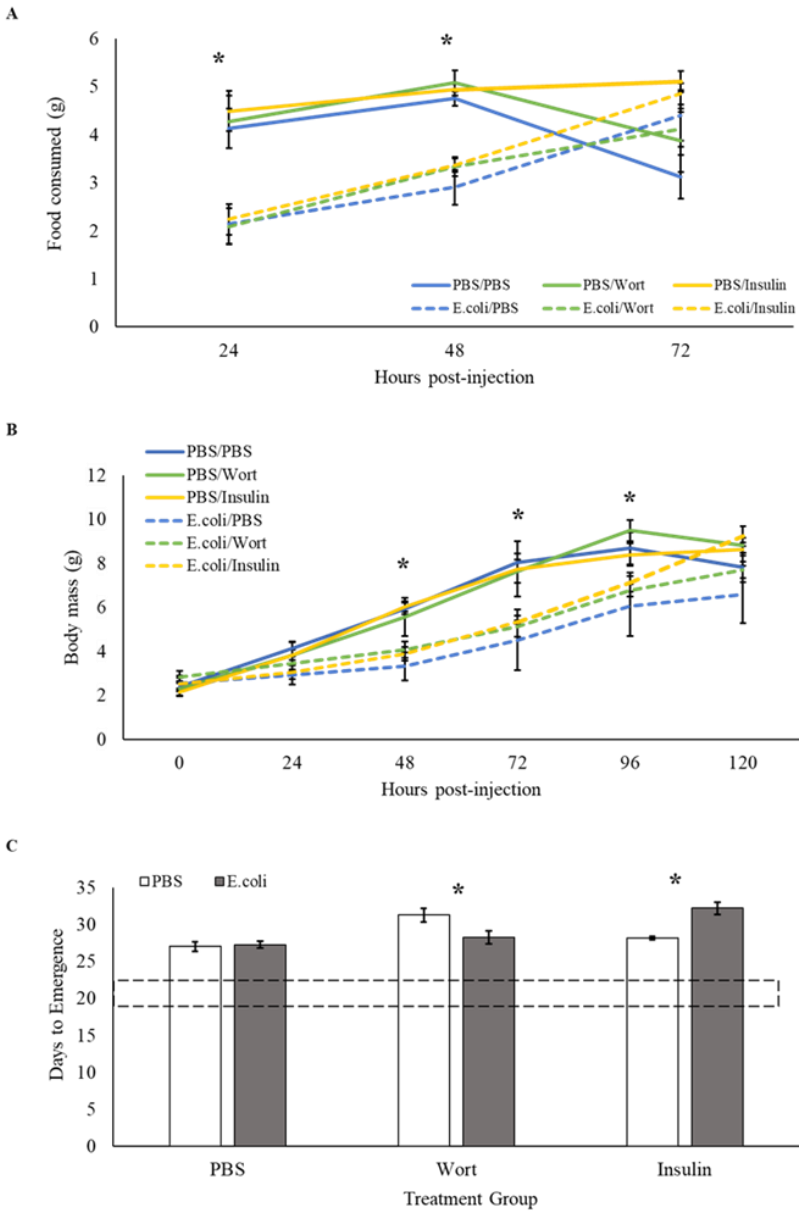

Figure 10. Insulin signaling manipulation in 5th instar *M. sexta* during SIA. Effects of manipulating insulin signaling by either inhibition (wortmannin) or increasing (bovine insulin) on A) feeding and B) body mass. Dashed line indicated the average days to emergence of 19-23 days. * indicate significant differences between PBS and *E. coli* groups.

Table 2. Summary statistics from Kruskal-Wallis test for the detection of significant differences in gene expression levels between treatment groups in brain samples. Summary statistics for each gene tested with nCounter in brain samples log transformed means ± standard error for each treatment group, one-way test ChiSquare (χ^2) values, degrees of freedom (df), and p-values (p). * $p < 0.05$

Gene	Control	PBS	SIA	χ^2	df	*p*
3-phosphoinositide-dependent protein kinase 1	4.92±0.04	5.04±0.15	6.85±0.10	5.60	2	0.06
bombyxin-related peptide A-like	1.12±0.11	1.08±0.14	1.48±0.55	0.08	2	0.95
bombyxin-related peptide B-like	1.68±0.87	2.89±0.91	1.81±0.58	1.15	2	0.56
bombyxin-related peptide B-like 2	0.89±0.34	0.96±0.16	1.34±0.24	2.48	2	0.28
dSOR1 (ERK)	5.82±0.07	5.84±0.08	5.84±0.23	0.00	2	1.00
embryonic polarity protein dorsal-like	6.94±0.04	6.78±0.07	6.99±0.07	3.46	2	0.17
forkhead box protein O	4.63±0.09	4.73±0.04	4.99±0.09	5.95	2	0.05
glycogen synthase kinase-3 beta-like	4.40±0.03	4.24±0.02	4.05±0.16	5.60	2	0.06
gonadotropin-releasing hormone (AKH) receptor	3.64±0.68	2.69±0.65	2.54±1.11	1.06	2	0.58
gonadotropin-releasing hormone receptor	2.95±0.18	3.61±0.17	2.91±0.37	5.60	2	0.06
GTP-binding protein Rheb homolog	5.19±0.06	5.26±0.12	5.22±0.11	0.62	2	0.73
insulin receptor substrate 1	3.93±0.06	3.82±0.09	5.27±0.11	5.95	2	0.05
insulin receptor-like	3.99±0.02	3.85±0.23	4.84±0.38	4.35	2	0.11
insulin-like growth factor 1 receptor	3.17±0.16	3.87±0.12	3.24±0.17	5.60	2	0.06
insulin-related peptide 1-like	3.42±0.08	4.15±0.55	3.83±0.18	1.68	2	0.42
insulin-related peptide 2-like	3.55±0.25	4.29±0.38	4.16±0.26	3.28	2	0.19
M. sexta serine/threonine-protein kinase Mtor	5.46±0.08	5.4±0.03	5.3±0.25	0.08	2	0.95
mitogen-activated protein kinase 1	5.43±0.06	5.56±0.06	6.05±0.09	6.48	2	**0.04***
mitogen-activated protein kinase p38b-like	6.52±0.05	6.46±0.09	6.26±0.07	4.35	2	0.11
myc proto-oncogene protein	4.27±0.09	4.68±0.08	5.27±0.06	7.20	2	**0.03***
myeloid differentiation primary response protein MyD88-like	5.50±0.02	5.19±0.11	5.35±0.17	3.20	2	0.20
NF-kappa-B inhibitor cactus	4.10±0.28	4.37±0.22	5.23±0.27	5.60	2	0.06
PTEN	4.64±0.05	4.79±0.05	4.11±0.18	7.20	2	**0.03***
PI3K catalytic subunit type 3	3.86±0.20	3.77±0.07	3.93±0.22	0.26	2	0.87
PI3K regulatory subunit gamma	5.52±0.14	5.41±0.14	5.28±0.12	2.22	2	0.32
protein eiger	7.15±0.11	6.90±0.11	6.36±0.15	6.48	2	**0.04***
protein kinase C	5.74±0.05	5.76±0.09	5.65±0.15	0.35	2	0.83
protein pellino-like	6.05±0.07	5.84±0.06	5.93±0.05	3.82	2	0.14
protein son of sevenless-like	5.10±0.01	5.01±0.13	4.85±0.25	0.80	2	0.67
protein spaetzle	4.78±0.08	4.89±0.13	4.85±0.04	1.06	2	0.5

Table 2. Summary statistics from Kruskal-Wallis test for the detection of significant differences in gene expression levels between treatment groups in brain samples (continued). Summary statistics for each gene tested with nCounter in brain samples log transformed means ± standard error for each treatment group, one-way test ChiSquare (χ^2) values, degrees of freedom (df), and p-values (p). * $p < 0.05$

Gene	Control	PBS	SIA	χ^2	df	*p*
protein toll-like	1.96±0.06	2.39±0.29	4.41±0.57	5.95	2	0.05
ras-like protein 2	6.56±0.05	6.51±0.09	6.46±0.05	2.22	2	0.32
serine/threonine-protein kinase mTOR-like	3.60±0.15	4.09±0.22	3.37±0.60	2.48	2	0.28
stress-activated protein kinase JNK	4.43±0.10	4.46±0.20	4.08±0.28	1.86	2	0.39
tyrosine-protein kinase hopscotch	4.72±0.05	4.59±0.05	4.70±0.07	2.48	2	0.28

Table 3. Summary statistics from Kruskal-Wallis test for the detection of significant differences in gene expression levels between treatment groups in fat body samples. Summary statistics for each gene tested with nCounter in fat body samples log transformed means ± standard error for each treatment group, one-way test ChiSquare (χ^2) values, degrees of freedom (df), and p-values (p). * $p < 0.05$* $p < 0.05$, ** $p < 0.01$

Gene	Control	PBS	SIA	χ^2	df	*p*
3-phosphoinositide-dependent protein kinase 1	4.62±0.17	4.14±0.10	6.91±0.11	12.74	2	**0.001****
bombyxin-related peptide A-like	1.23±0.33	0.41±0.11	0.78±0.15	7.01	2	**0.030***
bombyxin-related peptide B-like	0.96± 0.39	0.38±0.18	0.57±0.19	1.47	2	0.478
bombyxin-related peptide B-like 2	1.27±0.15	0.36±0.15	0.9±0.15	8.32	2	**0.015***
dSOR1 (ERK)	4.58±0.06	4.75±0.04	5.86±0.36	12.44	2	**0.002****
embryonic polarity protein dorsal-like	6.42±0.16	5.94±0.12	6.61±0.21	7.04	2	**0.029***
forkhead box protein O	4.85±0.17	4.39±0.12	5.0±0.08	7.16	2	**0.027***
glycogen synthase kinase-3 beta-like	3.33±0.41	2.28±0.17	2.91±0.29	6.77	2	**0.034***
gonadotropin-releasing hormone (AKH) receptor	7.20±0.37	6.46±0.12	5.34±0.34	7.15	2	**0.028***
gonadotropin-releasing hormone receptor	3.62±0.71	2.29±0.19	2.19±0.22	2.50	2	0.285
GTP-binding protein Rheb homolog	5.52±0.56	4.15±0.18	4.79±0.32	5.64	2	0.059
insulin receptor substrate 1	4.40±0.38	3.36±0.13	5.53±0.25	10.72	2	**0.005****
insulin receptor-like	3.53±0.17	3.00±0.11	4.61±0.09	13.07	2	**0.002****
insulin-like growth factor 1 receptor	0.85±0.31	0.22±0.12	0.38±0.20	3.81	2	0.149
insulin-related peptide 1-like	1.19±0.23	1.07±0.25	0.92±0.07	1.85	2	0.395
insulin-related peptide 2-like	0.82±0.33	0.27±0.10	0.48±0.18	1.76	2	0.414
M. sexta serine/threonine-protein kinase Mtor	4.19±0.25	4.28±0.11	4.80±0.17	6.32	2	**0.043***
mitogen-activated protein kinase 1	4.69±0.28	4.20±0.07	5.05±0.23	6.63	2	**0.036***
mitogen-activated protein kinase p38b-like	6.79±0.56	5.67±0.12	5.99±0.36	1.29	2	0.523
myc proto-oncogene protein	4.96±0.42	4.9±0.15	4.55±0.29	1.83	2	0.400
MyD88-like	4.80±0.16	4.61±0.11	5.71±0.27	11.70	2	**0.003****

Table 3. Summary statistics from Kruskal-Wallis test for the detection of significant differences in gene expression levels between treatment groups in fat body samples (continued). Summary statistics for each gene tested with nCounter in fat body samples log transformed means ± standard error for each treatment group, one-way test ChiSquare (χ^2) values, degrees of freedom (df), and p-values (p). * $p < 0.05$* $p < 0.05$, ** $p < 0.01$

Gene	Control	PBS	SIA	χ^2	df	*p*
NF-kappa-B inhibitor cactus	3.50±0.35	2.70±0.10	5.35±0.30	12.44	2	**0.002****
PTEN	5.07±0.50	4.02±0.15	3.47±0.29	7.94	2	**0.019***
PI3K catalytic subunit type 3	3.87±0.23	3.34±0.13	3.65±0.18	3.48	2	0.175
PI3K regulatory subunit gamma	5.61±0.50	4.60±0.20	4.76±0.40	2.53	2	0.282
protein eiger	5.8±0.11	6.07±0.15	6.49±0.13	9.02	2	**0.011***
protein kinase C	5.11±0.47	3.99±0.16	4.67±0.31	4.16	2	0.124
protein pellino-like	5.23±0.36	4.33±0.10	6.21±0.34	9.90	2	**0.007****
protein son of sevenless-like	4.14±0.16	4.28±0.10	4.36±0.08	0.94	2	0.625
protein spaetzle	3.96±0.26	3.63±0.11	4.37±0.24	6.72	2	**0.035***
protein toll-like	1.46±0.25	1.98±0.06	3.27±0.34	10.85	2	**0.004****
ras-like protein 2	6.51±0.48	5.43±0.12	5.83±0.33	1.90	2	0.387
serine/threonine-protein kinase mTOR-like	2.87±0.17	3.00±0.02	2.99±0.10	1.35	2	0.509
stress-activated protein kinase JNK	3.71±0.24	3.90±0.07	3.87±0.12	0.44	2	0.803
tyrosine-protein kinase hopscotch	5.28±0.29	4.65±0.14	5.77±0.31	7.09	2	**0.028***

Gene expression of major signaling pathways

To determine what genes are involved in SIA, I measured expression levels of target genes of the IIS, TOLL, MAPK, and JAK-STAT pathways. Expression levels were compared among control, vehicle-injected, and SIA groups in fat bodies and brains. For both tissue samples, SIA affected gene expression compared to both control groups. Overall, gene expression in brains changed very little in response to treatments (Table 2), with only 11% of genes showing significant differences compared to control groups. In the fat body during SIA, 57% of genes differed significantly, with most of the immunity-related genes varying from control groups (Table 3).

Insulin signaling (IIS) pathway regulation during SIA

Expression levels of several genes varied between fat bodies from caterpillars in the SIA group compared to the PBS group. Expression of *insulin receptor-like* (InR) was three-fold higher in the SIA group than the PBS group (Fig. 11A, Z = 3.54, p = 0.001). This three-fold increase was also observed for *insulin receptor substrate* 1 (IRS) (Fig. 11B, Z = 3.20, p = 0.004), *phosphoinositide-dependent protein kinase* 1(PDK) (Fig. 11C, Z = 3.487, p = 0.0015), and *forkhead box protein O* (FOXO) (Fig. 11D, Z = 2.51, p = 0.036). *Manduca sexta serine/threonine-protein kinase (*mTOR) was differentially expressed between SIA and PBS groups in fat body samples, but Bonferroni pairwise comparisons did not detect statistically significant differences among the groups (Fig. 11E, all p values > 0.089). *Myc proto-oncogene* (myc) expression increased four-fold in the SIA group relative to the Control group in brain samples (Fig. 11F, Z = 2.53, p = 0.034). Some genes were down-regulated in SIA groups. Expression levels for PTEN in the fat body was three-fold lower in the SIA group in comparison to the Control group (Fig. 12A, Z = -2.74, p = 0.0183), and four-fold lower in comparison to the PBS group in the brain (Fig. 12A, Z = -2.53, p = 0.034). SIA had no effect on fat body expression levels in three genes, but control and PBS groups differed for *bombyxin-related peptide A-like* (Fig. 12B, Z = -2.56, p = 0.0317), *bombyxin-related peptide B-like* 2 (Fig. 12C, Z = -2.79, p = 0.016), and *glycogen synthase kinase-3 beta-like* (GSK3B) (Fig. 12D, Z = -2.41, p = 0.047). The remainder of the IIS target genes tested did not differ between any treatment groups or by tissue.

SIA upregulation of Toll pathway targets

Because IIS is known to be decreased by activation of the Toll pathway, I measured expression levels for its target genes. For all of the target genes measured in the Toll pathway,

the vehicle-injected (PBS) group differed from the bacterial-injected (SIA) group. The Toll pathway is activated when the ligand *spaetzle* binds to the receptor. In the fat body, expression levels for spaetzle were two-fold higher in the SIA group compared to the PBS group (Fig. 13A, $Z = 2.52$, $p = 0.036$). The receptor, *toll-like*, differed significantly with the SIA group being higher than the Control group (Fig. 13B, $Z = 3.13$, $p = 0.005$), but not the PBS group ($Z = 2.17$, $p = 0.090$). Fat bodies of SIA caterpillars also had higher expression levels compared to the PBS groups for all targets downstream of spaetzle, including *myeloid differentiation primary response protein MyD88-like* (MyD88) 1 (Fig. 13C, $Z = 3.25$, $p = 0.003$), *pellino-like* (Fig. 13D, $Z = 3.09$, $p = 0.006$), *NF-kappa-B inhibitor* (cactus) (Fig. 13E, $Z = 3.43$, $p = 0.0018$), and *embryonic polarity protein dorsal-like* (dorsal) (Fig. 13F, $Z = 2.46$, $p = 0.042$). There were no significant differences for any Toll pathway genes in the brain. Gene expression for the MAPK-JNK-p38 pathways showed a similar trend with SIA groups being significantly different, but not enough genes were measured in this pathway to make any clear connections (Fig. B2).

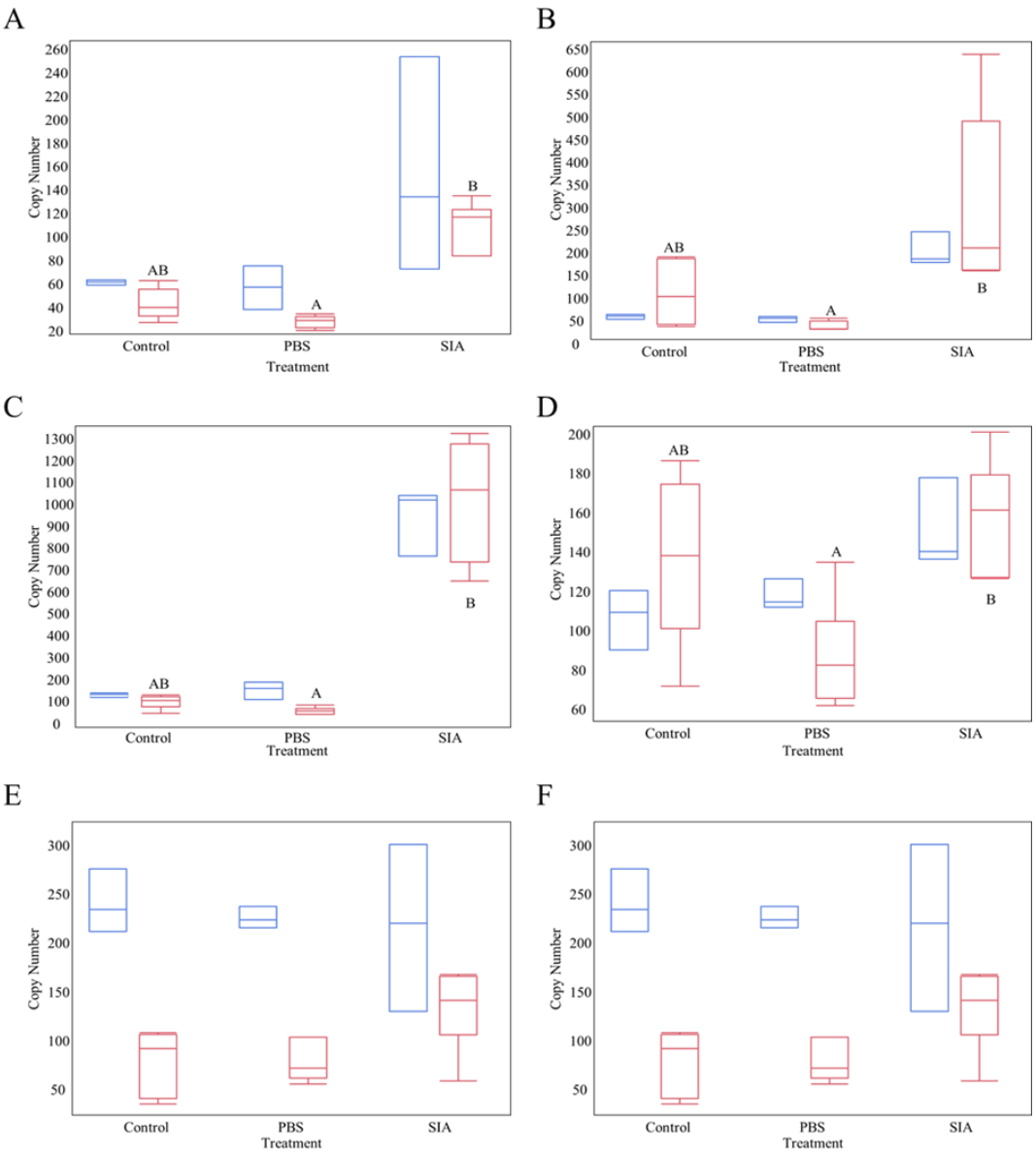

Figure 11. IIS targets that were significantly upregulated by SIA. Genes that were upregulated by SIA in the insulin signaling pathway such as A) *InR*, B) *IRS*, C) *PDK*, D) *FOXO*, E) *mTOR*, F) *myc* in fat body (red) and brain samples (blue). Different letters indicate significant differences between groups in fat body samples. There were no significant differences in brain samples.

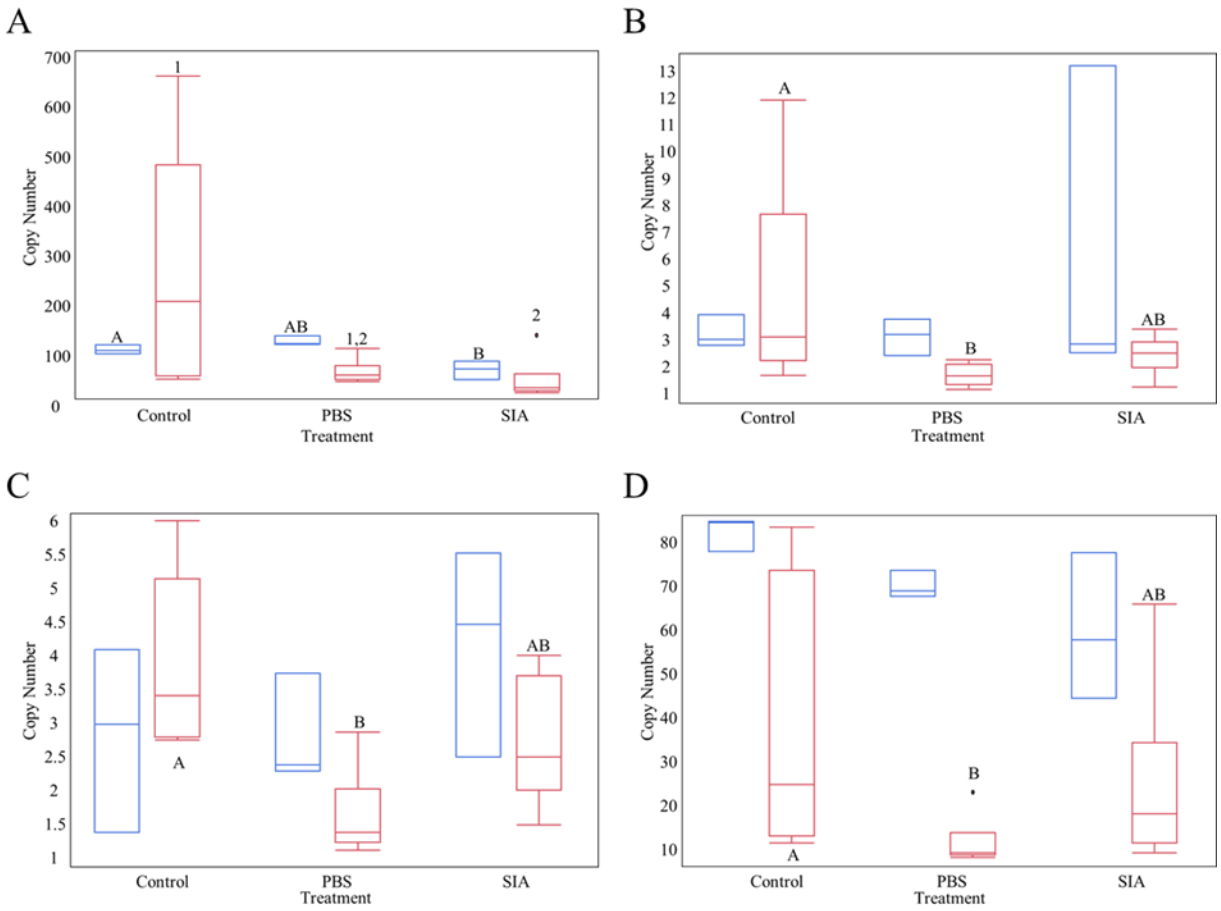

Figure 12. IIS targets that were significantly downregulated by SIA or showed no change. Genes that were downregulated by SIA in the insulin signaling pathway such as A) *PTEN*, or that showed no response to SIA like B) *bombyxin-related peptide A-like*, C) *bombyxin-related peptide B-like* 2, and D) *GSK3B* in fat body in fat body (red) and brain samples (blue). Different letters indicate significant differences between groups in fat body samples. There were no significant differences in brain samples.

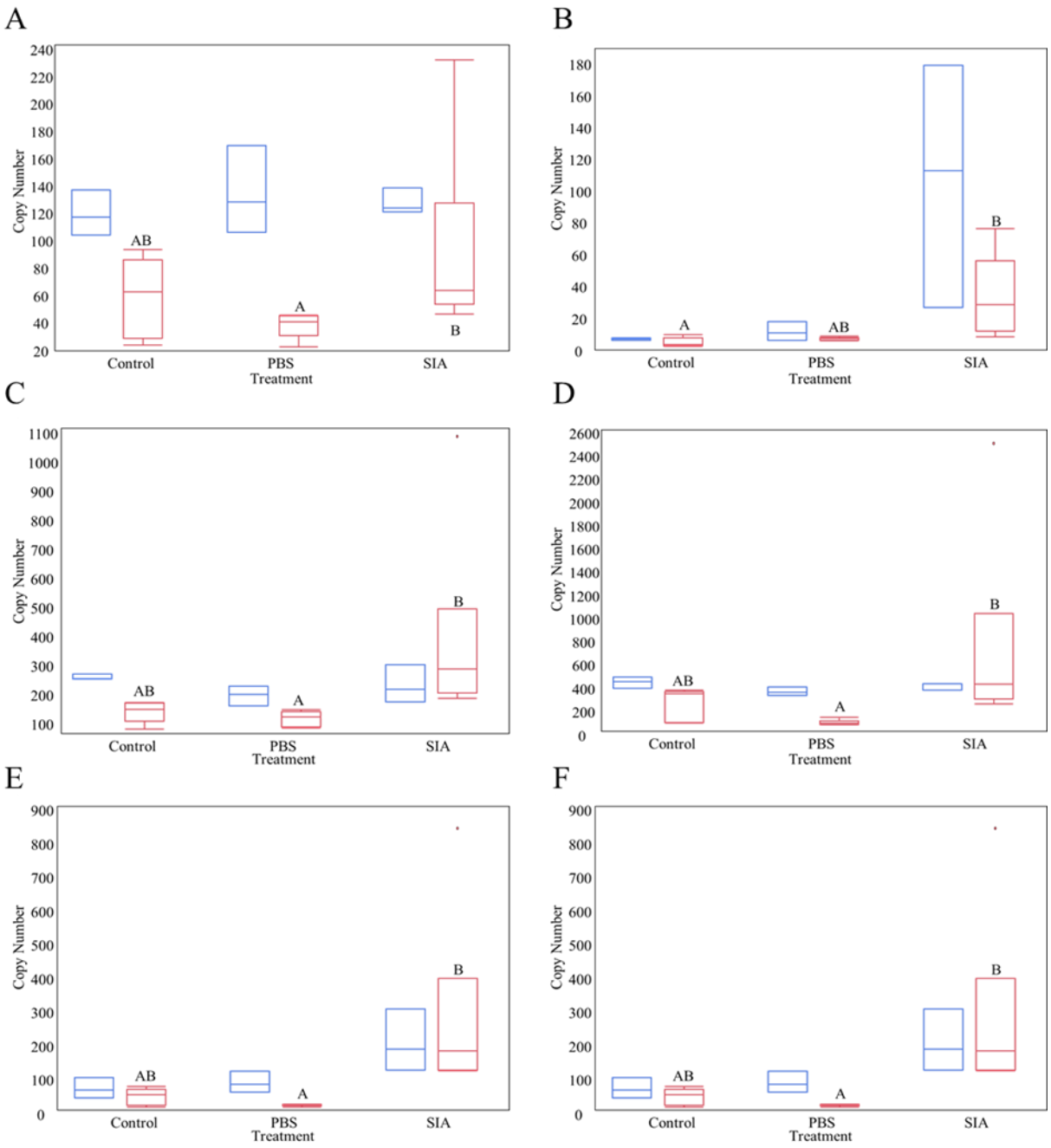

Figure 13. Gene expression of Toll pathway targets. Expression of Toll pathway targets A) *spaetzle*, B) *toll-like*, C) *MyD88*, D) *pellino-like*, E) *cactus*, and F) *dorsal* in fat body (red) and brain samples (blue). Different letters indicate significant differences between groups in fat body samples.

Discussion

Sickness-induced anorexia (SIA) has been studied in insects such as the cricket *Gryllus texensis* and the tobacco hornworm *M. sexta* (Adamo, 2005; Adamo et al., 2010; Adamo et al., 2016; Adamo et al., 2007). In addition to previous studies, larvae of *M. sexta* are a reliable model of SIA, with repeatable metrics, shown here in decreased food consumption (Fig. 10A, Fig. B3). However, I saw no effect of increasing or decreasing insulin on larval growth during SIA. To confirm that dosage was not the issue, I performed additional studies, and feeding was not impacted by any dose of insulin, regardless if the larvae were exhibiting SIA or not (Fig. B3). Interestingly, insulin manipulation resulted in long-term effects on developmental timing. This provides strong support for the null hypothesis that insulin signaling does not directly regulate SIA. Because of this finding, the second part of this study focused on determining what genes may be involved in SIA, by measuring gene expression of key regulatory pathways in fat bodies and brains. Fat body samples had the most significant changes in expression for insulin and immunity-related genes, which was expected because the fat body produces humoral response molecules like AMP and relays nutritional information to the brain. However, in response to SIA, both brain and fat body tissues showed more differences in immunity-related genes than insulin target genes, providing further support for the null hypothesis that insulin signaling does not directly regulate SIA.

Interestingly, my results suggest that the IIS pathway is being suppressed downstream at *FOXO*. My previous findings of SIA induced by injecting heat-killed bacteria resulted in increased AMP production (Booth et al., 2015), therefore I focused on pathways that utilize the IIS pathway to induce AMP production. IIS pathway target *FOXO* is an important transcription factor for stress tolerance and AMP production (Becker et al., 2010), therefore *FOXO* expression

was predicted to be upregulated in this study. In *Drosophila* and *B. mori*, levels of *FOXO* were increased by reduced *AKT* phosphorylation, leading to elevated AMP production (DiAngelo et al., 2009; Roth et al., 2018; Zhang et al., 2018). In the current study, along with an upregulation of *FOXO,* I also observed an increase in its upstream inhibitors such as *insulin-like receptor* and *phosphoinositide-dependent protein kinase 1 (PDK)*, meaning that suppression of the signaling cascade is could be occurring between *PDK* and *FOXO*. Under extreme starvation, *FOXO* can also feedback and regulate *InR* expression, but not its activation, to increase sensitivity to insulin in *Drosophila* (Junger et al., 2003; Puig et al., 2003). This is predicted to be a preparatory mechanism that allows for a fast response to insulin upon refeeding. Interestingly, both FOXO and InR were upregulated in response to SIA, suggesting that this feedback loop also occurs in *M. sexta*. These findings suggest that mechanisms utilizing FOXO to regulate insulin signaling are also being utilized in response to the cessation of feeding of SIA.

Furthermore, suppression of the IIS pathway and subsequent upregulation of *FOXO* may be an important mechanism for increasing stress tolerance and perhaps regulating immune responses. In *Drosophila*, *FOXO* regulates the IIS pathway in response to oxidative stress via adipokinetic hormone (AKH) (Bednarova et al., 2015). If this mechanism is also conserved in *M. sexta,* I would have expected my observed increase in *FOXO* expression level to be correlated with an increase in AKH signaling. However, my results showed a three-fold decrease of *gonadotropin-releasing hormone (AKH) receptor* in the SIA group relative to the Control group in fat body samples (Fig. B4, $Z = -2.54$, $p = 0.033$). Additionally, gene expression of protein kinase C (*PKC),* which targets *FOXO,* did not change in response to treatment or by tissue, unlike results seen in previous studies where *PKC* is needed for AKH-mediated immune responses (Bednarova et al., 2013, 2015). Together, these data suggest that AKH does not induce

FOXO to induce AMP production during SIA in *M. sexta.* Since I only measured expression levels of the *AKH* receptor and *PKC*, more studies are needed to definitively tell whether AKH signaling plays a role in suppressing insulin signaling during SIA to induce AMP production. AMP production can also be induced via *FOXO* by the Toll pathway. A previous study showed that Toll activation during a bacterial infection led to reduced insulin signaling at the *AKT* level, releasing its inhibition of *FOXO* (DiAngelo et al., 2009; Dionne et al., 2006).

SIA caterpillars in my study had twice the level of gene expression for several target genes of the Toll pathway compared to the control groups (Fig. 13) and increased *FOXO* expression (Fig. 11), indicating that the Toll pathway may indeed be suppressing the IIS pathway in response to SIA. Based on my results and previous studies, I propose a mechanism involving the insulin signaling pathway in SIA (Fig. 14). During SIA, increased expression of the Toll pathway may lead to suppression of the IIS pathway, resulting in higher *FOXO* expression. The elevated levels of *FOXO* then lead to increased *InR* expression. With elevated *InR* levels, it is assumed that insulin sensitivity is also increased as seen in *Drosophila* (Junger et al., 2003; Puig et al., 2003). This increase in sensitivity would explain why I observed no effect of SIA on the number of days to prepupation in my growth experiment (not shown), indicating larvae were able to quickly resume growth and development comparable to control groups.

The increase in InR and insulin sensitivity has been previously shown in response to starvation (Junger et al., 2003; Puig et al., 2003). Additionally, in *B. mori*, starvation upregulates the transcription of IIS pathway genes such as *InR*, *IRS*, *PDK*, and *PTEN* (Liu et al., 2010), similar to my results during SIA (Fig. 11), and others like *PTEN* (Fig. 12) being downregulated during SIA. My study also showed no effect on expression of both regulatory and catalytic subunits of *PI3K* (Table 2, Table 3) as seen in *B. mori* (Liu et al., 2010). The increase in IIS

pathway transcription are in response to starvation phenotypes during pupation and molting in *B. mori*. In my model, *M. sexta*, growth and differentiation of imaginal disks are controlled by ILP (Koyama et al., 2008; Nijhout et al., 2007), and genomic data shows that ILP expression slightly increases during pupation (Kanost et al., 2016), similar to *B. mori*. Therefore my results seem to match that IIS transcription is upregulated in response to starvation.

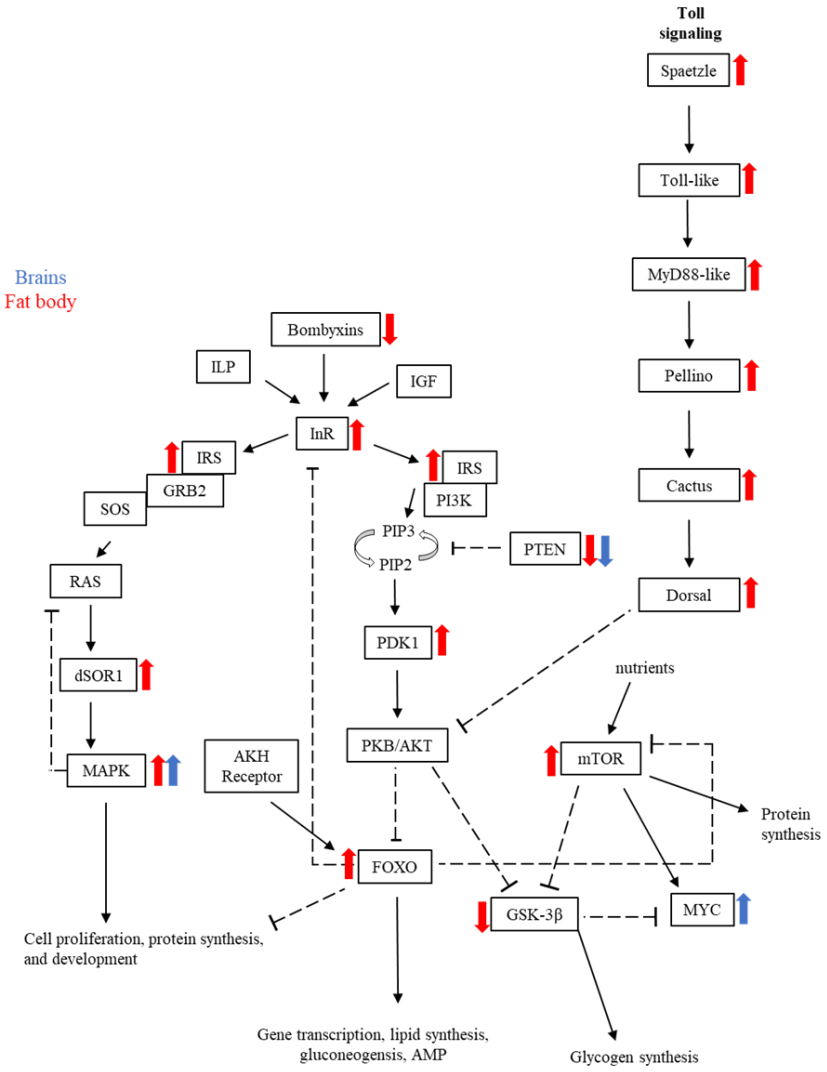

Figure 14. Model pathway showing study results of SIA on gene expression in *M. sexta*. Model pathway showing interconnection between the insulin and immune response pathways. Results for brain (blue) and fat body (red) samples are shown by either up arrows indicating upregulation, or down arrows indicating downregulation. No arrow indicates there were no significant differences in the SIA group of either tissue. See discussion for explanation of significantly different genes.

My study used heat killed bacteria, therefore, it makes sense that it induces responses similar to those during starvation rather than responses to a bacterial infection. Using both heat-killed and live *Serratia marcescens* to induce SIA in *M. sexta* result in negative effects on weight gain, but effects are exaggerated with live bacteria (Adamo et al., 2007). This could mean that heat-killed bacteria result in negative effects that induce different immune responses.

The focus of this study was to determine the role of insulin signaling in SIA. I hypothesized that insulin signaling regulated the cessation of feeding associated with SIA. My results support the idea that insulin signaling is suppressed in order to allow energy to be reallocated towards immune function, although whether that is achieved through Toll signaling requires further testing. The hormone, 20-hydroxyecdysone, has been suggested to reduce food consumption to induce a state of starvation (Liu et al., 2010; Wang et al., 2010), resulting in elevated mRNA expression of IIS pathway components during molting and pupation (Liu et al., 2010). Although I did not measure 20E, my results with similar increased expression in IIS pathway targets may indicate an involvement of 20E in the cessation of feeding during SIA. The IIS and 20E network has been previously shown (Deng et al., 2018; Hou et al., 2012; Keshan et al., 2017; Roy et al., 2007; Wang et al., 2010), therefore 20E being involved in SIA would make sense. Although there have been several studies on SIA in various taxa, the molecular pathways mediating the benefits of SIA on immunity have not been elucidated. Altered feeding behavior during infection or parasitism has been overlooked and vastly understudied, despite being a behavior that is conserved across taxa and play a role in host defenses (Hite et al., 2020). With advances in technology, insect genomes are becoming more readily available, especially for non-model insects. These advances have drastically changed not only the number of questions we can answer, but the type of questions we can answer about the physiology of non-model insects.

Although common mechanisms may be used between insect species, there are still several differences that call for research to be done in a variety of insects.

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CHAPTER 4. EFFECTS OF TEMPERATURE VARIATION ON EXPRESSION OF GENES IN THE INSULIN SIGNALING PATHWAY IN OVERWINTERING *MEGACHILE ROTUNDATA*

Abstract

Diapause is a non-feeding state that many insects undergo to survive the winter months. During these months of overwintering, how nutrition is regulated is still unclear. Diapausing insects are exposed to environmental stressors including temperature stress, but responding can be energetically costly. With fixed resources, overall metabolism and insulin signaling are maintained at low levels, but whether those pathways change in response to seasonal temperature fluctuations remains unknown. The focus of this study is to determine 1) how insulin signaling varies throughout diapause and 2) if that variation changes in response to temperature. To test the hypothesis that insulin signaling is responsible for allocating energy in response to fluctuations in temperature during overwintering months, alfalfa leafcutting bees, *Megachile rotundata*, were overwintered in either a lab setting at a constant 4˚C or in the field in naturally fluctuating temperatures, and then measured expression of genes in the insulin pathway, including key players and targets of the IIS pathway. Control genes were chosen from a previous RNAseq study and validated using quantitative real-time polymerase chain reaction (qRT-PCR). Subsets of each RNA sample were used for both qPCR and nCounter analysis. Using the same samples and genes of interest allowed us to compare results from these two techniques with the previous results from RNAseq. Although using qPCR only one gene, *MAPK14B_1*, differed with temperature, nCounter analysis detected 17 genes that varied significantly by temperature treatments. I predicted that IIS gene expression would change over time in response to fluctuating temperatures. When looking at how insulin signaling changed over time, the *insulin-*

like receptor, *GAB2*, *MAPK14B*, all three cell cycle genes (*cyclins D, E, G*), and *samui* differed. However, of the genes that differed during overwintering months, only 3 were targets of the insulin signaling pathway. The other genes, *cyclins* and *samui*, responded as expected by changing over time due to their roles in regulating development. To answer my second question, I compared gene expression levels between temperature treatments at each month for a given gene. The results showed that, again all three cyclins (*D, E, G*), *FOXO*, *GAB2*, *insulin-like receptor* and *insulin-receptor like*, *MAPK14B*, *PI3K* regulatory subunit, and *samui* were significantly different between constant and fluctuating temperature groups at several time points. Overall my results showed that a few key targets of the insulin signaling pathway along with growth regulators change during overwintering, suggesting that only cell cycle regulators, and not the insulin signaling pathway as a whole, change across the phases of diapause. When looking at the effects of temperature on gene expression, I observed significantly more differences in expression of target genes of the insulin signaling pathway, indicating temperature does influence insulin signaling. It is possible that bees kept in a constant temperature did not initially decrease their insulin signaling due to a lack of environmental temperature cues. With differences seen between temperature treatment groups, these findings indicate that constant temperatures like those used in agricultural storage protocols, lead to different expression profiles and possibly different phenotypes for alfalfa leafcutting bees.

Keywords: Insect, Insulin signaling, Diapause, alfalfa leafcutting bee, Development, Differential gene expression, Nutrition, Metabolism, Pollinator

Introduction

To survive winter months, insects living in the temperate zone undergo diapause, a state of developmental dormancy (Denlinger, 2002). For many insects, diapause is a non-feeding state,

meaning all feeding to collect sufficient energy reserves for the winter and subsequent period of development must be done prior to its initiation (Denlinger, 2002; Hahn and Denlinger, 2007; Lehmann et al., 2016). Energy reserves not only help insects survive the winter, but also provide the initial energy to insects coming out of post-diapause quiescence. Insects emerging from diapause need to have fuel to replenish reserves and prepare for further development or reproduction (Hahn and Denlinger, 2011). Energy expenditure during overwintering is minimized by a decrease in metabolic rate and cell-cycle arrest, key characteristics indicating the onset of diapause (Danks, 1987; Denlinger, 2002; Hahn and Denlinger, 2011). The drop in metabolism during diapause is crucial for conserving energy reserves and enables insects to survive long periods without feeding. Because diapause can span from months to years (Hahn and Denlinger, 2007, 2011), both the collection of initial reserves and subsequent reduction of energy use are important.

The main energy reserves of diapausing insects consist of three groups of macronutrients: amino acids, carbohydrates, and lipids (Hahn and Denlinger, 2007, 2011). Lipids are primarily stored as triacylglycerides in the main storage organ, the insect fat body (Canavoso et al., 2001). The fat body is the primary site for diapausing and non-diapausing insects to store and synthesize carbohydrates in the form of glycogen (Danks, 1987). Glycogen can be quickly broken down and transported between tissues as the major blood sugar disaccharide, trehalose (Arrese and Soulages, 2009). Trehalose and amino acids also aid in cold and desiccation resistance, and many diapausing insects accumulate higher concentrations of trehalose and amino acids in their blood (Boctor, 1981; Kostal et al., 2016; Kostal and Simek, 1995; Lefevere et al., 1989; Li et al., 2002). Amino acids are stored in proteins in the fat body specialized for diapause maintenance, post-diapause development, and reproduction called hexamerins (Burmester, 1999; Denlinger,

2002; Denlinger et al., 2005). All three major macronutrients are required prior to diapause initiation, but the mechanism behind nutrient sensing and regulation during diapause is not entirely clear.

Many studies point to the insulin signaling pathway as a possible mediator of nutrient regulation and sensing (Arsic and Guerin, 2008; Defferrari et al., 2016a; Defferrari et al., 2016b; DiAngelo et al., 2009; Li et al., 2016; Taguchi and White, 2008). The insulin/IGF-1 signaling (IIS) pathway is well known to regulate growth, development, and both carbohydrate and lipid metabolism in mammals (Bates et al., 2013; Dupont and Holzenberger, 2003; Dupont and Scaramuzzi, 2016; Saltiel and Kahn, 2001). Research on insect insulin-like peptides (ILP) has shown they play an important role in diapause phenotypes, such as halted reproduction (Sim and Denlinger, 2009), reserve accumulation (Satake et al., 1997), metabolic depression (Hahn and Denlinger, 2011), and enhanced stress-tolerance (Matsunaga et al., 2016; Sim and Denlinger, 2013; Wu and Brown, 2006). With limited energetic resources during this non-feeding stage, diapausing insects can be more susceptible to low temperature stress.

Further evidence shows that IIS pathway targets also aid in mechanisms dealing with low temperature stress. During diapause, some insects are exposed to environmental stressors (Bosch et al., 2010; Bradshaw and Holzapfel, 2010; Fliszkiewicz et al., 2012; Hoback and Stanley, 2001; Musolin, 2007; Sgolastra et al., 2016; Sgolastra et al., 2011; Sgolastra et al., 2012). Most insects are freeze avoidant and prevent freezing through upregulation of heat-shock proteins, production of cryoprotectants, and modification of cytoskeletal elasticity (Denlinger, 1991; Rinehart et al., 2007; Sinclair et al., 2003; Toxopeus and Sinclair, 2018). These mechanism(s) for cold resistance overlap with targets of the IIS pathway in *Caenorhabditis elegans* (Savory et al., 2011) and *Drosophila melanogaster* (Broughton et al., 2005). Oxidative stress resistance by

manganese-superoxide dismutase, is known to be regulated at the gene level by the IIS downstream target, *Forkhead box transcription factor* (FOXO) (Honda and Honda, 1999; Kops et al., 2002). In response to high levels of reactive oxygen species (ROS), *FOXO* induces resistance to low temperature, oxidative stress, and pathogenic infections in the pupae of the moth *Helicoverpa armigera* (Zhang et al., 2017). Closing the gap in knowledge about how IIS is involved in diapause is crucial for understanding the physiological and biochemical changes that occur in insects during this sensitive period. This knowledge could also be used to improve the care of agriculturally important pollinators such as *Megachile rotundata*.

To understand how IIS is involved in regulating diapause, a starting point would be looking at changes in gene expression of the pathway during this developmental stage. Traditionally, this is done using quantitative polymerase chain reaction (qPCR), which has limitations, especially in non-model organisms (Huggett et al., 2005; Nolan et al., 2006). In addition to general limitations, such as variations in reverse transcription amplification, primer efficiencies, and inconsistent use of control genes, finding control genes for non-model organisms can be expensive and time-consuming (Bustin, 2004; Huggett et al., 2005; Nolan et al., 2006). Despite these limitations, their use continues, because lack of evidence and support for new techniques can be risky in non-model organisms. A multiplex approach, NanoString, can overcome some of the limitations of qPCR, but only a handful of studies have utilized this technique with insects (Ammeux et al., 2016; Choi et al., 2014; Epstein et al., 2017; Fulga et al., 2015; Keith et al., 2019; Lai et al., 2019; Sandler and Stathopoulos, 2016; Schell et al., 2017; Shiao et al., 2015; Shiao et al., 2013; Sun et al., 2018; Sung et al., 2013; West and Silverman, 2018; Winkler et al., 2017; Zheng et al., 2018).

The focus of this study is to determine 1) how insulin signaling varies throughout diapause and 2) how insulin signaling changes in response to temperature. I hypothesize that IIS is responsible for allocating energy reserves in response to temperature. To test this hypothesis, I measured mRNA expression of key players and targets of the IIS pathway in the alfalfa leafcutting bee, *M. rotundata*. Previous RNAseq studies using *M. rotundata* provide a broader view of how other pathways change during diapause, making it the ideal species in which to test this hypothesis.

Materials and methods

Experimental design

Samples collected during a previous experiment were used for both qPCR and nCounter analysis (Yocum et al., 2018). Briefly, *Megachile rotundata* were reared in an on-farm facility in Utah, USA in the summer of 2010. Adults were released into an alfalfa field (Logan, UT, 41°47′37.04″N; 112°8′18.35″W), and offspring were placed into treatment groups in October of 2010. Brood cells from straws pulled between June 30th and July 19th were considered early diapause. Early diapause bees were placed into one of two overwintering treatment groups, a lab setting at a constant 4-5˚C in darkness (Constant), or a field setting where insects were exposed to naturally fluctuating temperatures (Fluctuating), as described in Yocum et al. (2018). Temperatures outside and inside the field shelter were recorded with a HOBO Datalogger (Onset Computer Corp., Bourne, MA, USA). Prepupae overwintered in the field were exposed to temperatures ranging from -18˚C (January 2011) to 35˚C (May 2011) (Fig. S1;Yocum et al.).

Sample preparation

Each month, individual bees were chosen from both field and lab temperature treatments and flash-frozen in liquid nitrogen. Prepupae were ground in liquid nitrogen, and RNA was

extracted with TRIzol (Invitrogen, Life Technologies, Grand Island, NY, USA) according to manufacturer's instructions. RNA pellets were stored in -80˚C under absolute ethyl alcohol until used in this study. RNA samples from all months (November, December, January, February, March, April, May, June) and temperature treatments were used. RNA pellets were taken from -80˚C and ethanol was removed. Pellets were washed in 70% ethanol and dried before being resuspended in 30 µl of DEPC-treated water. RNA concentration was quantified using both a NanoDrop 1000 Spectrophotometer (V3.8 Thermo Fisher Scientific, Wilmington, DE, USA) and a Qubit 4 Fluorometer (Invitrogen, Life Technologies, Grand Island, NY, USA). Six bees per treatment group were used for qPCR, and 4 of those 6 were also used for nCounter analysis for November, January, March, and May.

Quantitative real-time PCR

Genes were chosen to span the IIS pathway from receptor to downstream targets to observe any changes in its regulation (Table E1). Transcript files from a previous Illumina study (Yocum et al., 2018) were mined for target gene sequences and aligned to the MROT_1.0 genome assembly with NCBI's BLASTn (v2.8.1+) (Zhang et al., 2000). Transcript variants for each gene were aligned using BioEdit (v7.0.5) (Hall, 1999). Primers for target and reference genes were designed using Primer Quest Tool from Integrated DNA Technologies (Coralville, IA, USA) for conserved regions across all variants (Table E2). Multiple primers were designed for genes that had various conserved regions. Illumina transcript data were screened for potential reference genes. The first ten genes with the lowest change in expression over time and between treatment groups were tested. Reference gene stability was analyzed using geNorm in qbase+ software (Biogazelle, Ghent, Belgium), and the geometric mean of the three most stable genes was used for normalization of gene expression. RNA samples from November, January, March

and May were used for qPCR and diluted to a target concentration of 280 ± 50 ng/µL. RNA samples were treated with DNase I followed by cDNA synthesis with Super Script III first strand synthesis system for RT-PCR (Invitrogen, Life Technologies, Grand Island, NY, USA). cDNA samples were diluted 1:10 for qPCR. A pooled sample (referred to as RT+) was used to run dissociation plates and all calibration reactions on reference and experimental qPCR plates. qPCR was conducted with SYBR Green I Master Mix on a LightCycler 480 (Roche, Indianapolis, IN, USA). Plate designs utilized the sample maximization method and three inter-run calibrators (IRC) to account for plate-to-plate variation. Samples were run in biological and technical triplicates at custom cycling parameters. The reactions were incubated at 95°C for 30 seconds, followed by 45 cycles of 55°C for 10 seconds, 70°C for 35 seconds, 95°C for 5 seconds, with an extension time of 65°C for 60 seconds, followed by melting curve analysis. Technical reps that did not fall within 1 amplification cycle of each other were excluded from analysis. The ΔΔCt method was used to calculate fold change, and values were base-10 log transformed.

NanoString nCounter

A custom probe codeset for *M. rotundata* was designed and manufactured using the same genes of interest as described above (Table E3) (NanoString Technologies Inc., Seattle, WA, USA). Because the multiplex technique allows for measurement of up to 800 genes, I included more genes from the insulin pathway, proto-oncogenes that are downstream of the insulin pathway, and genes involved in cell cycle regulation to monitor development during overwintering (Table E3). RNA pellets from November through June were resuspended as described above, diluted to a target concentration of 20 - 50 ng/µL, and loaded into a 96-well skirted plate. Plates were shipped on dry ice to the University of Minnesota Genomics Center

(Minneapolis, MN) for processing with nCounter Analysis System (NanoString Technologies Inc.). Data were normalized to the geometric mean of 10 reference genes.

Statistical analysis

JMP Pro software (v.14.0, SAS Institute Inc., 2018, Cary, NC, USA) and SAS (v.9.4 SAS/STAT 15.1, SAS Institute Inc., 2018, Cary, NC, USA) were used for statistical analyses. Hierarchical cluster analyses with a Ward-linkage method and constellation plots were performed with JMP Pro (v.14.0, SAS Institute Inc., 2018, Cary, NC, USA). Constellation plots arrange the individual genes as endpoints, and each cluster joins as a new point, with lines drawn that represent membership in gene expression. The circle identifies the root of the tree and the longer lines represent greater distance between clusters, indicating increasing dissimilarity between expression. The line length values are meaningful only with respect to each other, and the axis scaling, orientation of points, and angles of the lines are arbitrary.

Initial exploration of the data showed that there was a strong correlation between gene copy number (nCounter) or gene expression level (qPCR) and month. Even though the samples were independent observations, the month-to-month expression levels showed a strong correlation pattern that needed to be properly assessed in the statistical model. To do this, a random coefficient growth curve model (RCGCM) was used to model the linear relationship between copy number or gene expression level and the interaction of month and temperature for each gene (Vonesh, 2012). Each gene's regression line was determined by the gene's random intercept and random slope, with separate population intercept and slope also fit to the constant and fluctuating temperatures for each gene, respectively. The MODEL statement contains the response variable, Copy number or Gene Expression Level, equal to the fixed main effects of Temperature and Month as continuous regressors, and their interaction. The RANDOM

statement is utilized to instruct the procedure to treat the intercept and slope of the Month (as a continuous numeric) as random effects with an unstructured variance-covariance matrix. This was achieved by fitting the RCGCM in the MIXED procedure described above for each Gene and utilizing the ESTIMATE statement to calculate differences in intercepts (Main Temperature contrast) and differences in slopes (interaction of Temperature and Month/Time). Differences for intercepts and slopes between groups were calculated by subtracting estimates for the constant treatment group from estimates for the fluctuating treatment group. The delta values for slope and intercept estimates are reported. Negative values indicate that the fluctuating temperature treatment group were lower than the constant temperature group, and positive values indicate that they were higher than the constant temperature group. Due to the factorial type design, degrees of freedom were corrected using the Kenward-Roger approximation. A difference in intercept indicates a significant difference between temperature treatments in the month of November, while a difference in slope indicates a significant difference between temperature treatments over time. Post-hoc analyses of intercepts and slopes were conducted by comparing upper and lower confidence limits between treatment groups at each month. Limits that did not overlap were considered significantly different with 95% confidence. P-values less than 0.05 were considered significant. Gene expression levels that were below 1 were considered down regulated. Gene expression level and copy number means +/- SEM and 95% CI are reported.

Results

Quantitative real-time PCR

qPCR results showed little differences in gene expression of IIS pathway targets, with the only significant differences seen in the constant temperature treatment group, not supporting my hypothesis that IIS gene expression changes in response to temperature (Table 4). There was one

gene, *insulin-like growth factor 1* (IGF), that showed different expression patterns from other genes in all four months (Fig. 15). I also looked at overall changes in gene expression and saw that gene expression levels clearly separated into two clusters: November/March and December/January (Fig. 16). However, greater differences were seen in the Constant temperature treatment group. My second aim was to determine if gene expression of the insulin signaling pathway changed in response to temperature. Looking at individual genes by treatment groups (Fig. 17) showed that IGF was still most dissimilar for both treatments. Although the remaining genes showed no pattern between temperature treatment groups, there was a separate cluster for just the constant temperature treatment that consisted of *InR*, *MAPK1*, *mTOR_1*, *MYC*, and *PDK* genes (Fig. 17). *MAPK14B*_1, *MAPK14B*_2, and *mTOR_2* were the only genes out of the 8 measured that differed significantly by either temperature treatment or an interaction between treatment and time (Table 5). The intercept was significantly different for these genes, indicating that the fluctuating temperature treatment group were lower than the constant temperature group in the month of November (Fig. 18: *MAPK14B_1*: estimate = -0.49, DF = 48, p = 0.02; *MAPK14B_2*: estimate = -0.49, DF = 48, p = 0.005; *mTOR_2*: estimate = -0.38, DF = 47, p = 0.04). Post-hoc analysis for November samples, showed that bees overwintered in constant temperature had approximately 30% higher *MAPK14B_1* mean expression levels compared to those in fluctuating temperatures (Constant: 1.32±0.146; Fluctuating: 1.01±0.106). Despite having significant differences in intercepts, post hoc analysis did not identify differences in mean expression levels between bees in different treatments for *MAPK14B_2* and *mTOR_2* in November.

Table 4. Estimates table for qPCR results. Estimates table for intercepts and slopes of each gene tested with qPCR. Statistics include estimates, standard error (StdErr), Kenward-Roger adjusted degrees of freedom (DF), tValue, and p-value for the two-tailed test (Probt) for each gene's slope and intercept. P-values in bold are statistically significant.

Gene	Label	Estimate	StdErr	DF	tValue	Probt
FOXO	Intercept	-0.1581	0.182	48	-0.867323	0.3900
	Slope	0.00936	0.058	48	0.160303	0.8733
GSK3B_1	Intercept	-0.3054	0.191	48	-1.59772	0.1166
	Slope	0.0265	0.061	48	0.43274	0.6671
GSK3B_2	Intercept	-0.2503	0.150	47	-1.660214	0.1035
	Slope	0.02761	0.048	47	0.572359	0.5698
IGF	Intercept	-0.1583	0.264	44	-0.599061	0.5522
	Slope	0.03587	0.082	44	0.432706	0.6673
InR	Intercept	-0.5025	0.271	46	-1.852374	0.0703
	Slope	0.10562	0.085	46	1.242090	0.2204
MAPK1	Intercept	-0.5318	0.271	45	-1.955479	0.0567
	Slope	0.07286	0.084	45	0.858049	0.3954
MAPK14B_1	Intercept	-0.4339	0.179	48	-2.422457	0.0192
	Slope	0.04443	0.057	48	0.774492	0.4424
MAPK14B_2	Intercept	-0.4932	0.168	48	-2.927714	0.0052
	Slope	0.07428	0.053	48	1.37670	0.1749
MYC	Intercept	-0.3597	0.200	47	-1.795015	0.0790
	Slope	0.08192	0.063	47	1.284645	0.2052
PDK	Intercept	-0.2962	0.288	46	-1.025495	0.3104
	Slope	0.0115	0.091	46	0.126321	0.9000
PI3K_REG	Intercept	-0.3501	0.313	45	-1.118122	0.2694
	Slope	0.06661	0.101	45	0.657242	0.5143
mTOR_1	Intercept	-0.2615	0.184	47	-1.418075	0.1627
	Slope	0.02108	0.059	47	0.357266	0.7224
mTOR_2	Intercept	-0.3849	0.185	47	-2.077861	0.0432
	Slope	0.02726	0.058	47	0.462371	0.6459

Table 5. Summary statistics for genes that showed significantly different expression levels with qPCR. Summary statistics for each gene tested with qPCR include mean copy number (Mean copy num), standard deviation (Std dev), standard error (Std err), coefficient of variation (CV), lower and upper limits for 95% confidence intervals (CI) for each gene by temperature treatment (Temp) and month. Temperature treatments are fluctuating (fluc) and constant (con). Months are coded as November (1), December (2), January (3) and March (5).

Gene	Temp	Month	Mean fold-change	Std dev	Std err	CV	95% CI	
							Lower	Upper
FOXO	Fluc	1	0.598	0.3937	0.16	65.767	0.186	1.012
		2	0.631	0.7737	0.32	122.57	-0.181	1.443
		3	0.961	0.8440	0.35	87.76	0.076	1.847
		5	0.485	0.2328	0.10	47.99	0.241	0.729
	Con	1	0.433	0.3694	0.15	85.27	0.046	0.821
		2	0.343	0.2588	0.11	75.36	0.072	0.615
		3	0.388	0.0777	0.03	19.98	0.307	0.470
		5	0.411	0.3073	0.13	74.60	0.089	0.734
GSK3B_1	Fluc	1	0.164	0.0902	0.04	54.93	0.070	0.259
		2	0.253	0.1106	0.05	43.55	0.138	0.370
		3	0.255	0.0444	0.02	17.38	0.209	0.302
		5	0.151	0.0961	0.04	63.39	0.051	0.252
	Con	1	0.115	0.0810	0.03	70.30	0.030	0.200
		2	0.159	0.1450	0.06	91.10	0.007	0.311
		3	0.165	0.0900	0.04	54.26	0.071	0.260
		5	0.129	0.0976	0.04	75.22	0.027	0.232
GSK3B_2	Fluc	1	0.179	0.0634	0.03	35.28	0.113	0.246
		2	0.248	0.1274	0.05	51.32	0.115	0.382
		3	0.253	0.0247	0.01	9.75	0.223	0.284
		5	0.158	0.0755	0.03	47.63	0.079	0.238
	Con	1	0.126	0.0612	0.03	48.50	0.062	0.190
		2	0.171	0.1404	0.06	81.98	0.024	0.319
		3	0.174	0.1226	0.05	70.14	0.046	0.304
		5	0.150	0.1013	0.04	67.45	0.044	0.257
IGF	Fluc	1	0.006	0.0061	0.00	101.08	-0.000	0.012
		2	0.004	0.0029	0.00	76.54	-0.000	0.009
		3	0.008	0.0088	0.00	107.54	-0.001	0.017
		5	0.003	0.0019	0.00	67.93	0.000	0.005
	Con	1	0.003	0.0031	0.00	100.64	-0.000	0.006
		2	0.005	0.0042	0.00	79.08	0.000	0.011
		3	0.005	0.0023	0.00	46.76	0.002	0.008
		5	0.003	0.0016	0.00	59.63	0.001	0.004
InR	Fluc	1	0.082	0.0777	0.04	94.49	-0.041	0.206
		2	0.061	0.0638	0.03	103.86	-0.006	0.128
		3	0.047	0.0266	0.01	56.96	0.019	0.075
		5	0.029	0.0171	0.00	59.98	0.011	0.047

Table 5. Summary statistics for genes that showed significantly different expression levels with qPCR (continued). Summary statistics for each gene tested with qPCR include mean copy number (Mean copy num), standard deviation (Std dev), standard error (Std err), coefficient of variation (CV), lower and upper limits for 95% confidence intervals (CI) for each gene by temperature treatment (Temp) and month. Temperature treatments are fluctuating (fluc) and constant (con). Months are coded as November (1), December (2), January (3) and March (5).

Gene	Temp	Month	Mean fold-change	Std dev	Std err	CV	95% CI	
							Lower	Upper
InR	Con	1	0.038	0.0437	0.02	115.39	-0.008	0.084
		2	0.037	0.0414	0.02	112.49	-0.007	0.080
		3	0.053	0.0413	0.02	77.63	0.010	0.097
		5	0.034	0.0302	0.01	89.54	0.002	0.065
MAPK1	Fluc	1	0.065	0.0256	0.01	39.20	0.034	0.097
		2	0.133	0.0927	0.05	69.76	-0.015	0.280
		3	0.143	0.1542	0.06	107.58	-0.019	0.305
		5	0.034	0.0192	0.01	55.84	0.014	0.055
	Con	1	0.044	0.0360	0.02	82.74	0.006	0.081
		2	0.059	0.0438	0.02	74.22	0.013	0.105
		3	0.047	0.0176	0.01	37.11	0.029	0.066
		5	0.026	0.0153	0.01	60.19	0.009	0.042
MAPK14B_1	Fluc	1	0.100	0.0238	0.01	23.94	0.075	0.125
		2	0.108	0.0674	0.03	62.49	0.037	0.179
		3	0.136	0.1118	0.05	82.36	0.018	0.253
		5	0.033	0.0140	0.01	42.06	0.019	0.048
	Con	1	0.050	0.0164	0.01	32.99	0.033	0.067
		2	0.044	0.0280	0.01	63.25	0.015	0.074
		3	0.044	0.0179	0.01	41.28	0.025	0.062
		5	0.027	0.0195	0.01	73.05	0.006	0.047
MAPK14B_2	Fluc	1	0.194	0.1089	0.05	56.05	0.080	0.309
		2	0.225	0.1212	0.05	53.99	0.097	0.352
		3	0.458	0.4605	0.19	100.66	-0.026	0.941
		5	0.145	0.0722	0.03	49.91	0.069	0.221
	Con	1	0.079	0.0390	0.02	49.29	0.038	0.120
		2	0.109	0.0682	0.03	62.54	0.038	0.181
		3	0.139	0.0562	0.02	40.48	0.080	0.198
		5	0.134	0.0857	0.04	64.10	0.044	0.224
MYC	Fluc	1	0.020	0.0133	0.01	65.57	0.006	0.034
		2	0.045	0.0357	0.02	79.61	0.000	0.089
		3	0.039	0.0405	0.02	103.23	-0.003	0.082
		5	0.021	0.0059	0.00	28.00	0.015	0.027

Table 5. Summary statistics for genes that showed significantly different expression levels with qPCR (continued). Summary statistics for each gene tested with qPCR include mean copy number (Mean copy num), standard deviation (Std dev), standard error (Std err), coefficient of variation (CV), lower and upper limits for 95% confidence intervals (CI) for each gene by temperature treatment (Temp) and month. Temperature treatments are fluctuating (fluc) and constant (con). Months are coded as November (1), December (2), January (3) and March (5).

Gene	Temp	Month	Mean fold-change	Std dev	Std err	CV	95% CI	
							Lower	Upper
MYC	Con	1	0.015	0.0075	0.00	50.74	0.007	0.023
		2	0.017	0.0107	0.00	64.51	0.005	0.028
		3	0.023	0.0127	0.01	54.12	0.010	0.037
		5	0.031	0.0207	0.01	66.30	0.010	0.053
PDK	Fluc	1	0.139	0.1013	0.04	72.86	0.033	0.245
		2	0.158	0.1736	0.08	109.67	-0.057	0.374
		3	0.191	0.1865	0.08	97.59	-0.005	0.387
		5	0.079	0.0432	0.02	54.83	0.033	0.124
	Con	1	0.159	0.1729	0.08	108.45	-0.055	0.374
		2	0.086	0.0593	0.02	69.352	0.023	0.148
		3	0.064	0.0403	0.02	62.74	0.022	0.106
		5	0.070	0.0668	0.03	96.03	-0.001	0.140
PI3K_REG	Fluc	1	0.171	0.1591	0.07	92.89	0.004	0.338
		2	0.653	0.4652	0.21	71.22	0.076	1.231
		3	0.476	0.4730	0.21	99.48	-0.112	1.063
		5	0.196	0.1636	0.07	83.52	0.024	0.368
	Con	1	0.109	0.0965	0.04	88.69	0.008	0.210
		2	0.255	0.2216	0.09	87.00	0.022	0.487
		3	0.299	0.1679	0.07	56.18	0.123	0.475
		5	0.179	0.1478	0.07	82.71	-0.005	0.362
mTOR_1	Fluc	1	0.040	0.0221	0.01	55.43	0.017	0.063
		2	0.063	0.0259	0.01	41.26	0.036	0.090
		3	0.073	0.0106	0.00	14.54	0.060	0.086
		5	0.037	0.0188	0.01	51.14	0.017	0.056
	Con	1	0.031	0.0160	0.01	52.35	0.014	0.047
		2	0.041	0.0341	0.01	82.46	0.006	0.077
		3	0.043	0.0224	0.01	52.54	0.019	0.066
		5	0.035	0.0288	0.01	82.40	0.005	0.065
mTOR_2	Fluc	1	0.075	0.0498	0.02	66.03	0.023	0.128
		2	0.166	0.0706	0.03	42.57	0.078	0.254
		3	0.196	0.0797	0.03	40.64	0.113	0.280
		5	0.087	0.0471	0.02	54.45	0.037	0.136
	Con	1	0.059	0.0263	0.01	44.44	0.032	0.087
		2	0.059	0.0302	0.01	51.10	0.027	0.091
		3	0.056	0.0282	0.01	50.26	0.027	0.086
		5	0.070	0.0281	0.01	40.26	0.040	0.099

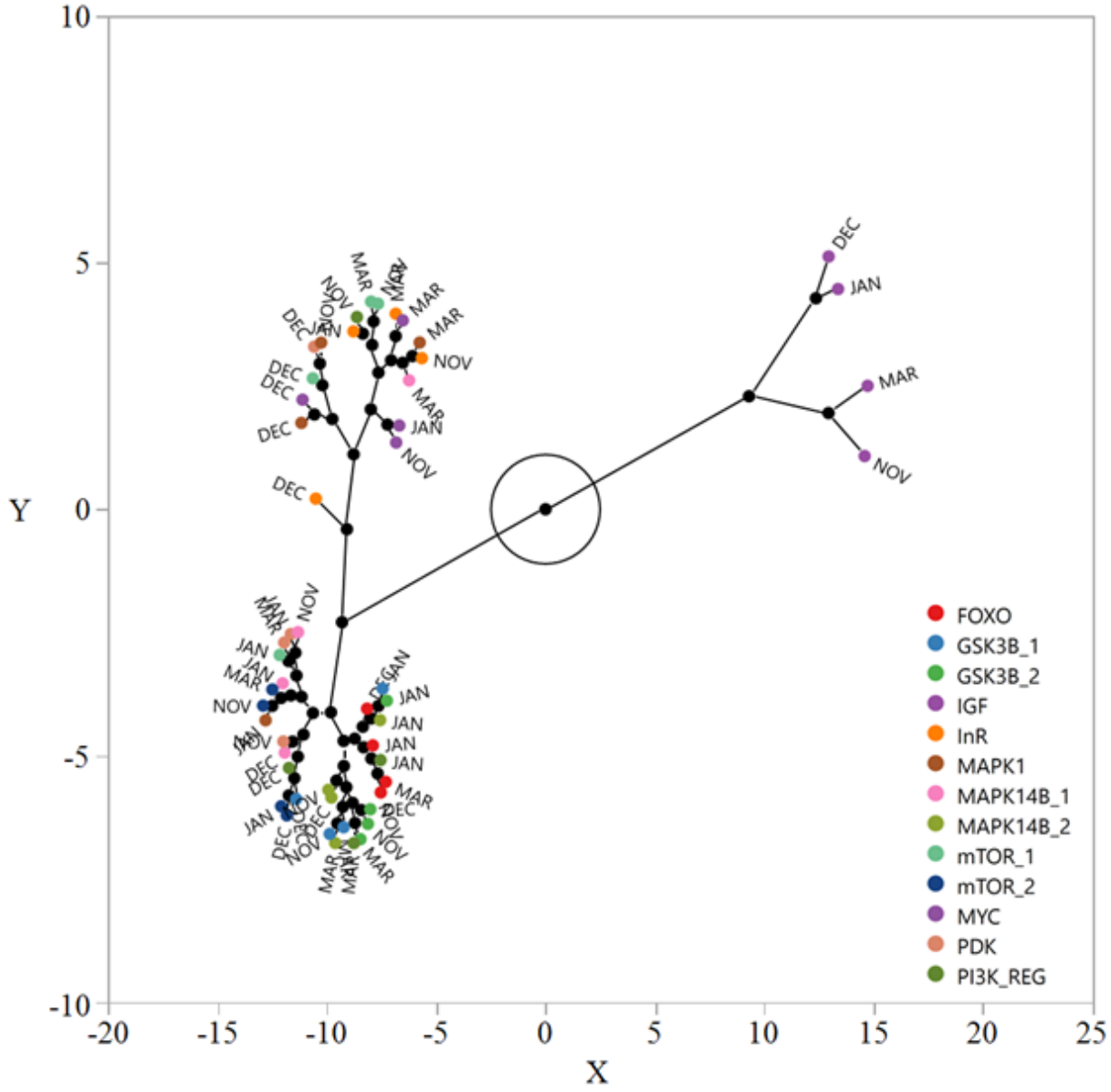


Figure 15. Constellation plot for gene expression measured by qPCR by month. Constellation cluster analysis for gene expression of individual genes by months. Each point represents the average gene expression for that gene at a given month. The farther the distance between points, the more dissimilar they are.

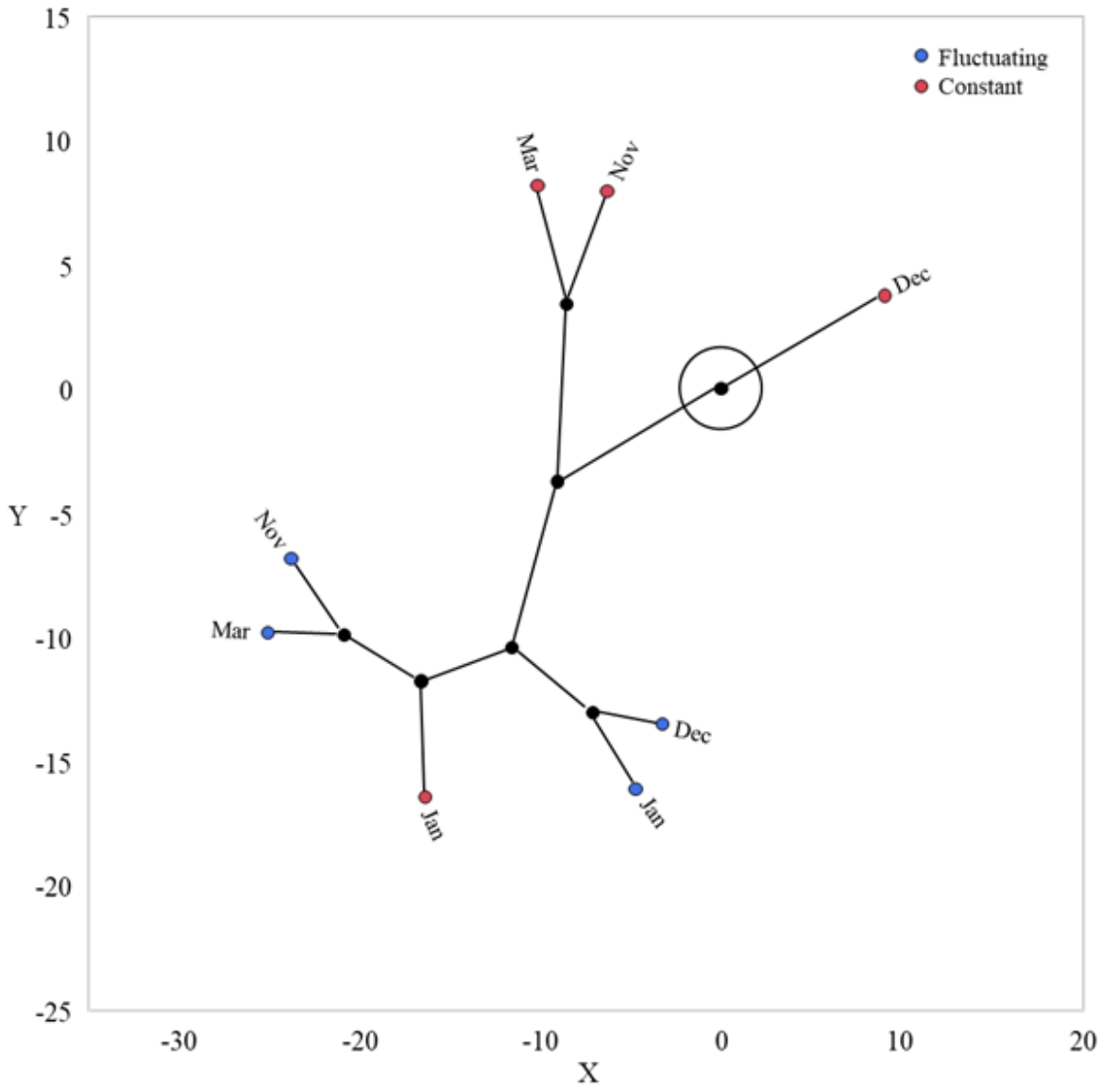


Figure 16. Constellation plot for qPCR gene expression by month for a given temperature treatment. Constellation cluster analysis for overall gene expression by temperature treatment by months. Each point represents gene expression for that month of the temperature treatment group. The farther the distance between points, the more different they are.

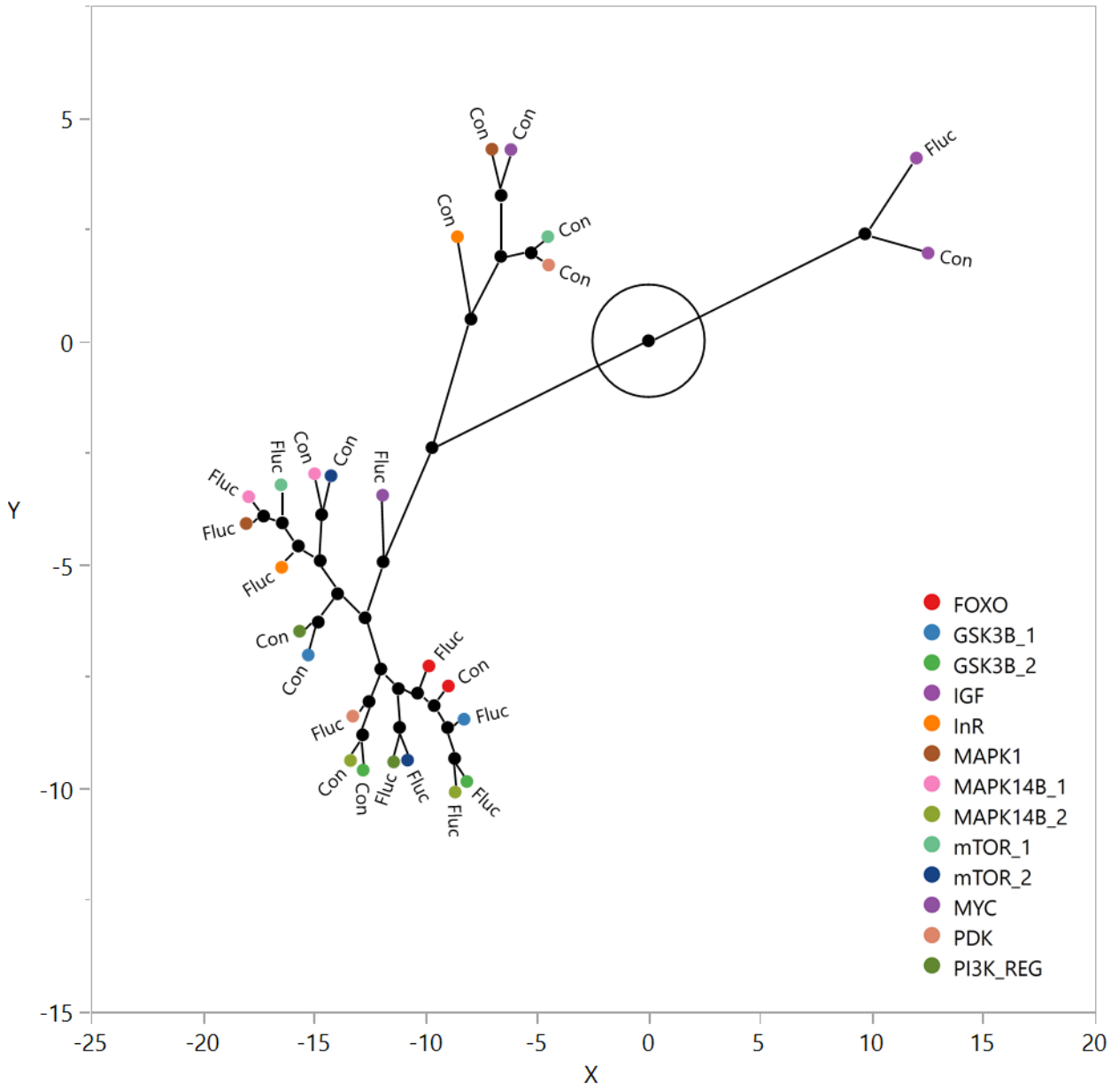


Figure 17. Constellation plot for gene expression measured by qPCR by temperature treatment for individual genes. Constellation cluster analysis for gene expression by fluctuating (fluc) and constant (con) temperature treatment for a given gene. Each point represents gene expression for that gene in the temperature treatment group. The farther the distance between points, the more dissimilar they are.

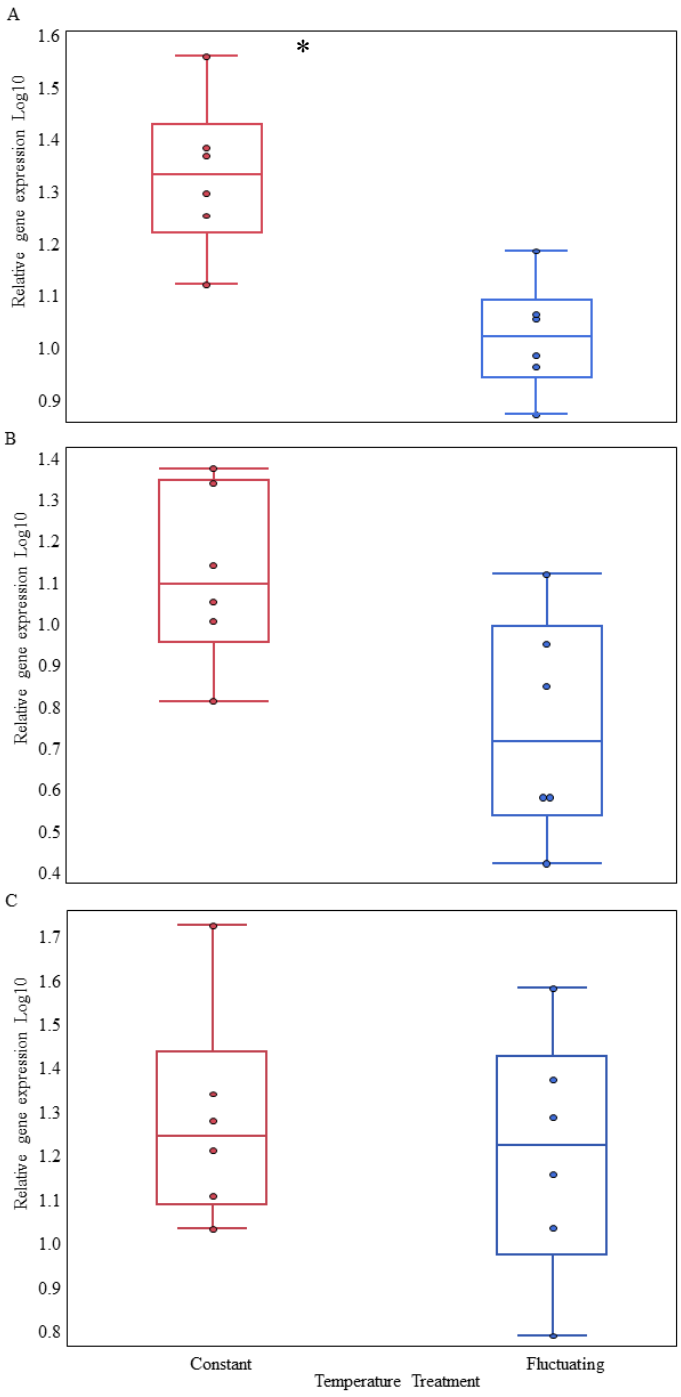


Figure 18. Relative gene expression in November for *MAPK14B*_1, *MAPK14B*_2, and *mTOR*_2. Relative gene expression levels for A) *MAPK14B_1*, B) *MAPK14B_2*, and C) *mTOR_2* genes in November. In the month of November, bees in constant temperature had higher *MAPK14B*_1 gene expression levels than those in fluctuating temperatures (estimate = -0.49, DF = 48, p = 0.02). Data shown is Log10 transformed. Post-hoc analyses for both showed no significance between treatment groups for *MAPK14B*_2 and *mTOR*_2.

NanoString nCounter

Since we were able to expand the number of genes and months tested, nCounter results more significant differences in genes over time and between treatment groups. Hierarchical cluster analysis showed expression patterns by month that were distinct to temperature treatments (Fig. 19). For the fluctuating temperature treatment group, gene expression separated into three clusters consisting of 1) Nov, Dec, 2) Jan, Feb, Mar, and 3) Apr, May, Jun. However, the constant temperature treatment group only had two clusters, 1) Nov-Dec and 2) Jan-Jun. Constellation plots showed that specific genes such as *samui*, *FOXO*, and *insulin-like receptor* showed different expression patterns between treatment groups and over time (Fig. 20A). However, *samui* showed more differences in expression as shown by months being separated. When looking at the effects of temperature on gene expression, again I saw the same three genes, *FOXO*, *insulin-like receptor*, and *samui* being main drivers (Fig. 20B). *Samui* was similar between treatments, but *FOXO* and *InR* showed significantly different clustering trends by treatments (Fig. 20B). Of the genes that were measured, 56% varied significantly, in either the slope, intercept, or both (Table 6).

IIS during overwintering

To determine how gene expression changes during overwintering, I used bees overwintered in field conditions with naturally fluctuating temperatures and compared monthly gene expression levels to T0 (November). Mean copy numbers of *cyclin D*, *cyclin E*, *cyclin G*, *GAB2*, *insulin-like receptor*, *MAPK14B*, and *samui* varied significantly throughout overwintering (Table 7). As overwintering progressed to spring, some cell cycle genes and the insulin receptor decreased in expression levels. *Cyclin D* mean copy number decreased by 44% from November to June (Fig. 21A; Nov: 36.39±5.5; Jun: 20.23±3.73). *Cyclin G* showed a similar pattern with a

34% decrease in mean copy number throughout overwintering (Fig. 21C; Nov: 280.73±15.90; Apr: 183.54±14.47). Expression of insulin-like receptor decreased by over half from November to June (Fig. 22B; Nov: 158.50±18.49; May: 53.20±5.89; Jun: 47.59±16.05), as did *MAPK14B* expression (Fig. 23D; Nov: 246.75±6.04; Jun:135.60±33.03). Other genes increased throughout the overwintering period. *Cyclin E* and *GAB2* expression levels increased by nearly 50% from November to January, and continued to show a 25% increase until spring (Fig. 21B; November: 32.49±5.20; January: 52.82±4.47; February: 66.14±7.63) (Fig. 23B; Nov: 68.37±6.06; Dec: 100.55±10.18; Jan: 137.15±10.17; Feb: 139.13±30.59; Mar: 140.76±35.13). *Samui* doubled every month until January and then remained high until April when it dropped 10-fold (Fig. 23F; Nov: 371.14±44.17; Dec: 664.62±21.59; Jan: 1363.43±205.52; Feb: 1464.55±138.57; Mar: 1800.33±463.10; Apr: 181.21±26.73).

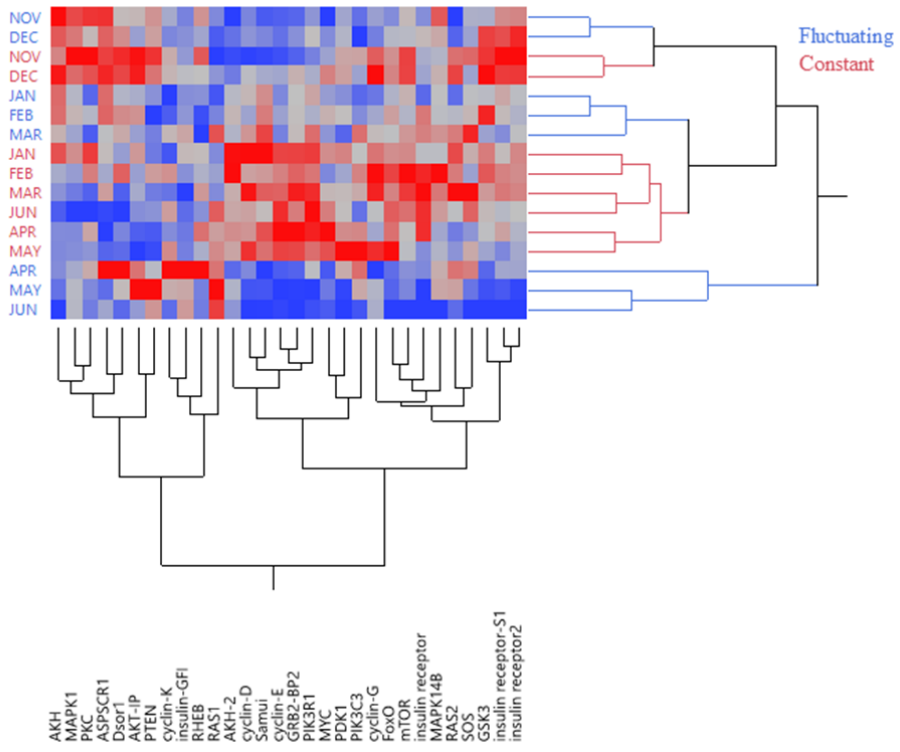


Figure 19. Hierarchical cluster of nCounter results. Two-way hierarchical cluster of nCounter results showing gene by month clustering.

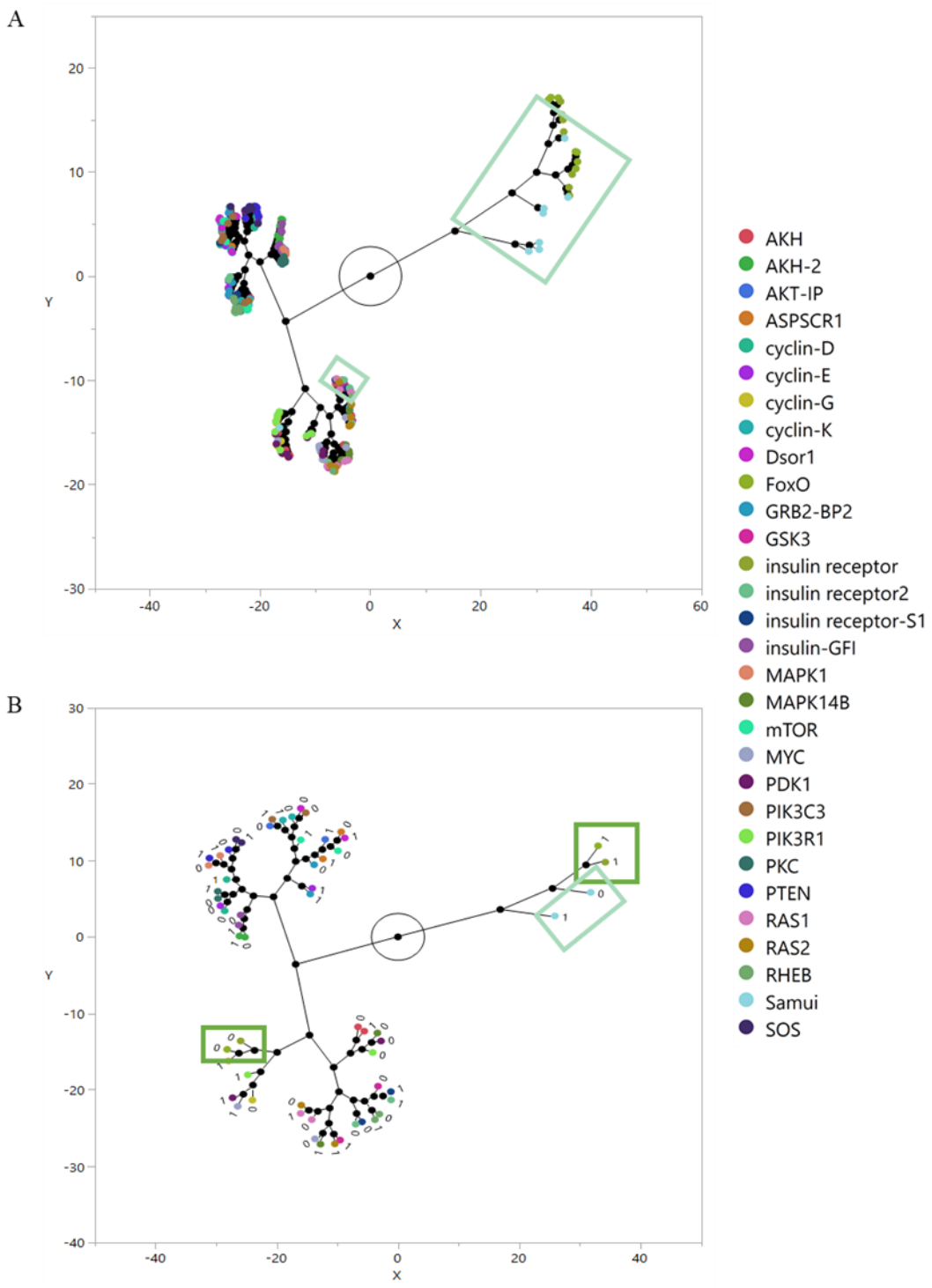


Figure 20. Constellation plot for nCounter gene expression. Constellation cluster analysis for genes by A) month and B) treatment group. Boxes in A represent samui gene clusters. Blue box in B represents *samui* clusters, while green boxes represent both *FOXO* and *InR* gene clusters. Treatment groups are labeled as fluctuating (0) and constant (1). Each point represents average gene expression for individual genes at a given A) month or B) temperature treatment group.

Table 6. Estimates table for nCounter results. Estimates table for intercepts and slopes of each gene tested with nCounter. Statistics include estimates, standard error (StdErr), Kenward-Roger adjusted degrees of freedom (DF), tValue, and p-value for the two-tailed test (Probt) for each gene's slope and intercept. P-values in bold are statistically significant.

Gene	Label	Estimate	StdErr	DF	tValue	Probt
(AKH) gonadotropin-releasing hormone II receptor	intercept	45.79178571	33.3379	64	1.37356	0.17437
	slope	-7.475744048	6.6019	64	-1.1324	0.26171
3-phosphoinositide-dependent protein kinase 1 (PDK1)	intercept	-23.18596047	35.7668	33.8378	-0.6483	0.5212
	slope	-13.85865023	9.33107	63.9154	-1.4852	0.1424
AKT-interacting protein-like	intercept	-43.85837489	10.8387	42.229	-4.0464	**0.00022**
	slope	11.04902498	2.38727	62.5661	4.6283	**1.9E-05**
GRB2-associated-binding protein 2	intercept	12.35568048	27.649	43.3936	0.44688	0.65719
	slope	-23.22836656	5.64971	62.8628	-4.1114	**0.00012**
GTP-binding protein Rheb homolog (RHEB)	intercept	-14.82613217	17.6783	35.0201	-0.8387	0.40735
	slope	0.992221936	4.48531	63.8347	0.22122	0.82563
Samui	intercept	239.7792857	330.243	64	0.72607	0.47044
	slope	-170.303869	65.3979	64	-2.6041	**0.01144**
adipokinetic prohormone type 2-like	intercept	-2.964464286	2.65994	64	-1.1145	0.26924
	slope	-0.103035714	0.52675	64	-0.1956	0.84554
cyclin-D	intercept	7.658303571	11.3524	64	0.6746	0.50236
	slope	-6.590178571	2.24811	64	-2.9314	**0.00467**
cyclin-E	intercept	13.59029365	17.4312	40.0143	0.77965	0.44018
	slope	-22.53686747	3.73643	63.6005	-6.0317	**9.1E-08**
cyclin-G	intercept	-102.1923618	20.4443	63.9392	-4.9986	**4.8E-06**
	slope	1.843043323	6.76212	63.1752	0.27255	0.78608
cyclin-K	intercept	-13.10182438	10.5206	33.6174	-1.2453	0.22162
	slope	2.688142921	2.50577	63.977	1.07278	0.2874
dual specificity mitogen-activated protein kinase kinase dSOR1 (ERK)	intercept	-14.83131043	8.33239	44.2098	-1.78	0.08196
	slope	4.469989935	1.68386	62.6266	2.65461	**0.01005**
forkhead box protein O	intercept	-1.235	66.4256	64	-0.0186	0.98522
	slope	-47.39229167	13.1542	64	-3.6028	**0.00062**
glycogen synthase kinase-3 beta-like	intercept	-7.066339286	15.0586	64	-0.4693	0.64048
	slope	-4.142619048	2.98205	64	-1.3892	0.16959
insulin receptor substrate 1	intercept	-6.707568265	24.0376	43.313	-0.279	0.78154
	slope	-5.050862319	4.91715	62.8848	-1.0272	0.30826
insulin receptor-like	intercept	103.956875	182.749	64	0.56885	0.57145
	slope	-82.610625	36.1897	64	-2.2827	**0.02578**
insulin-like growth factor I	intercept	-4.943381538	2.14636	23.5827	-2.3031	**0.03041**
	slope	2.314064502	0.81295	62.8864	2.84649	**0.00596**

Table 6. Estimates table for nCounter results. Estimates table for intercepts and slopes of each gene tested with nCounter (continued). Statistics include estimates, standard error (StdErr), Kenward-Roger adjusted degrees of freedom (DF), tValue, and p-value for the two-tailed test (Probt) for each gene's slope and intercept. P-values in bold are statistically significant.

Gene	Label	Estimate	StdErr	DF	tValue	Probt
insulin-like receptor	intercept	1.643392857	16.5877	64	0.09907	0.92139
	slope	-8.05985119	3.28486	64	-2.4536	**0.01688**
mitogen-activated protein kinase 1	intercept	-11.62571429	8.03486	64	-1.4469	0.1528
	slope	3.373422619	1.59114	64	2.12013	**0.03788**
mitogen-activated protein kinase 14B-like	intercept	33.48422931	12.3141	28.2158	2.71917	**0.01107**
	slope	-9.67307648	3.67719	56.165	-2.6306	**0.01098**
phosphatidylinositol 3-kinase catalytic subunit type 3	intercept	-23.02781929	8.20151	33.4646	-2.8078	**0.00826**
	slope	2.528402643	2.15867	63.9346	1.17128	0.24583
phosphatidylinositol 3-kinase regulatory subunit alpha (p85)	intercept	90.64870891	38.7744	37.4714	2.33785	**0.02484**
	slope	-41.2715591	8.63805	63.9022	-4.7779	**1.1E-05**
phosphatidylinositol 3/4/5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase (PTEN)	intercept	-12.93742548	5.63165	31.8353	-2.2973	**0.02833**
	slope	3.325966349	1.3925	63.875	2.38849	**0.01988**
protein L-Myc-1b	intercept	-19.12116071	37.9513	64	-0.5038	0.61611
	slope	-13.31758929	7.51548	64	-1.772	0.08115
protein kinase C	intercept	-8.645178571	4.51415	64	-1.9151	0.05995
	slope	1.300386905	0.89394	64	1.45468	0.15065
protein son of sevenless (SOS)	intercept	-1.832841902	5.67282	35.6865	-0.3231	0.74851
	slope	-0.197954078	1.41831	63.7754	-0.1396	0.88944
ras-like protein 1	intercept	-2.204411893	11.6861	32.4029	-0.1886	0.85155
	slope	3.031749535	2.85529	63.9151	1.0618	0.29232
ras-like protein 2	intercept	-65.89658571	12.8324	30.6111	-5.1352	**1.5E-05**
	slope	10.42929581	3.27178	63.7647	3.18766	**0.00222**
serine/threonine-protein kinase mTOR	intercept	-8.340877081	6.84603	29.8493	-1.2184	0.23263
	slope	-2.081295327	1.78793	63.6827	-1.1641	0.24873
tether containing UBX domain for GLUT4	intercept	-4.454910714	9.67512	64	-0.4605	0.64675
	slope	3.712410714	1.91596	64	1.93762	0.05708

Table 7. Summary statistics for genes with significantly different expression levels as determined by nCounter analysis. Summary statistics for each gene include mean copy number (Mean copy num), standard deviation (Std dev), standard error (Std err), lower and upper limits for 95% confidence intervals (CI) for each gene by temperature (temp) treatment and month. Months are coded as November (1), December (2), January (3), February (4), March (5), April (6), May (7), and June (8).

Gene	Temp	Month	Mean copy num	Std dev	Std err	95% CI Lower	Upper
AKT-interacting protein-like	Fluctuating	1	82.84	14.50	7.25	59.76	105.92
		2	72.50	19.70	9.85	41.15	103.85
		3	76.09	6.20	3.10	66.23	85.95
		4	89.01	15.54	7.77	64.27	113.74
		5	72.41	8.83	4.42	58.36	86.46
		6	94.39	18.70	9.35	64.64	124.14
		7	112.24	40.73	20.36	47.43	177.05
		8	71.00	22.25	11.12	35.60	106.39
	Constant	1	111.28	11.47	5.74	93.02	129.53
		2	109.33	44.26	22.13	38.90	179.76
		3	88.02	16.86	8.43	61.18	114.86
		4	72.44	13.59	6.79	50.82	94.07
		5	75.28	15.89	7.95	49.99	100.57
		6	55.46	15.76	7.88	30.38	80.54
		7	47.04	9.84	4.92	31.39	62.70
		8	67.97	17.81	8.91	39.62	96.31
GRB2-associated-binding protein 2	Fluctuating	1	68.37	6.06	3.03	58.72	78.01
		2	100.55	10.18	5.09	84.35	116.75
		3	137.15	10.17	5.09	120.96	153.33
		4	139.13	30.59	15.30	90.45	187.81
		5	140.76	35.13	17.56	84.87	196.66
		6	66.25	17.79	8.89	37.95	94.55
		7	40.10	13.01	6.51	19.39	60.81
		8	38.85	13.93	6.97	16.68	61.02
	Constant	1	65.45	12.78	6.39	45.11	85.79
		2	146.50	30.91	15.46	97.31	195.69
		3	209.37	51.02	25.51	128.18	290.56
		4	190.39	59.27	29.63	96.08	284.70
		5	230.46	65.34	32.67	126.49	334.42
		6	241.03	15.86	7.93	215.79	266.26
		7	207.34	81.66	40.83	77.39	337.28
		8	176.57	32.52	16.26	124.82	228.31

Table 7. Summary statistics for genes with significantly different expression levels as determined by nCounter analysis (continued). Summary statistics for each gene include mean copy number (Mean copy num), standard deviation (Std dev), standard error (Std err), lower and upper limits for 95% confidence intervals (CI) for each gene by temperature (temp) treatment and month. Months are coded as November (1), December (2), January (3), February (4), March (5), April (6), May (7), and June (8).

Gene	Temp	Month	Mean copy num	Std dev	Std err	95% CI Lower	Upper
Samui	Fluctuating	1	371.14	44.17	22.08	300.86	441.42
		2	664.62	21.59	10.80	630.26	698.97
		3	1363.43	205.52	102.76	1036.40	1690.46
		4	1464.55	138.56	69.28	1244.06	1685.04
		5	1800.33	463.09	231.55	1063.45	2537.21
		6	181.21	26.73	13.37	138.67	223.75
		7	338.32	82.67	41.34	206.78	469.87
		8	187.99	142.37	71.18	-38.55	414.52
	Constant	1	244.10	161.39	80.70	-12.71	500.91
		2	908.39	259.84	129.92	494.92	1321.85
		3	2138.95	373.87	186.94	1544.04	2733.87
		4	1293.48	648.80	324.40	261.09	2325.86
		5	1643.16	160.74	80.37	1387.39	1898.92
		6	1730.51	478.62	239.31	968.91	2492.10
		7	1536.32	396.30	198.15	905.71	2166.92
		8	1089.41	221.51	110.75	736.94	1441.87
cyclin-D	Fluctuating	1	36.39	5.47	2.73	27.69	45.08
		2	39.90	19.07	9.53	9.56	70.24
		3	52.10	11.71	5.85	33.47	70.72
		4	43.44	8.79	4.39	29.46	57.42
		5	58.57	22.62	11.31	22.58	94.56
		6	37.29	4.37	2.19	30.33	44.24
		7	25.25	11.74	5.87	6.57	43.93
		8	20.23	3.73	1.86	14.30	26.16
	Constant	1	21.15	4.29	2.14	14.33	27.98
		2	49.09	15.75	7.88	24.02	74.15
		3	85.51	15.19	7.59	61.34	109.67
		4	56.44	35.49	17.74	-0.03	112.91
		5	83.83	19.58	9.79	52.66	114.99
		6	58.80	25.70	12.85	17.90	99.69
		7	78.08	12.32	6.16	58.48	97.69
		8	56.26	18.27	9.14	27.18	85.33

Table 7. Summary statistics for genes with significantly different expression levels as determined by nCounter analysis (continued). Summary statistics for each gene include mean copy number (Mean copy num), standard deviation (Std dev), standard error (Std err), lower and upper limits for 95% confidence intervals (CI) for each gene by temperature (temp) treatment and month. Months are coded as November (1), December (2), January (3), February (4), March (5), April (6), May (7), and June (8).

Gene	Temp	Month	Mean copy num	Std dev	Std err	95% CI Lower	Upper
dual specificity mitogen-activated protein kinase kinase dSOR1 (ERK)	Constant	1	24.15	5.71	2.86	15.06	33.23
		2	68.33	31.63	15.82	18.00	118.67
		3	153.16	21.38	10.69	119.14	187.18
		4	117.97	57.25	28.63	26.86	209.07
		5	135.85	24.51	12.26	96.85	174.85
		6	185.52	34.32	17.16	130.92	240.13
		7	154.26	61.82	30.91	55.89	252.62
		8	166.91	44.25	22.12	96.50	237.32
cyclin-G	Fluctuating	1	280.73	15.90	7.95	255.42	306.04
		2	247.90	22.45	11.22	212.18	283.62
		3	240.26	21.02	10.51	206.81	273.72
		4	289.21	51.79	25.89	206.80	371.62
		5	316.66	84.08	42.034	182.88	450.44
		6	183.54	14.47	7.24	160.51	206.57
		7	301.80	97.5	48.78	146.57	457.02
		8	295.60	116.80	58.40	109.74	481.45
	Constant	1	359.56	39.68	19.84	296.42	422.70
		2	397.04	76.65	38.33	275.07	519.01
		3	348.95	39.18	19.59	286.60	411.29
		4	396.43	48.39	24.20	319.42	473.44
		5	386.45	59.02	29.51	292.54	480.36
		6	288.20	62.73	31.37	188.38	388.02
		7	381.79	118.24	59.12	193.64	569.93
		8	311.86	88.33	44.16	171.31	452.42
dual specificity mitogen-activated protein kinase kinase dSOR1 (ERK)	Fluctuating	1	77.36	11.99	6.00	58.28	96.44
		2	82.51	10.16	5.08	66.34	98.68
		3	74.82	15.39	7.69	50.33	99.30
		4	82.66	8.29	4.14	69.47	95.84
		5	79.25	16.16	8.08	53.54	104.95
		6	97.19	9.63	4.82	81.87	112.52
		7	77.35	21.60	10.80	42.98	111.72
		8	63.30	15.92	7.96	37.96	88.64

Table 7. Summary statistics for genes with significantly different expression levels as determined by nCounter analysis (continued). Summary statistics for each gene include mean copy number (Mean copy num), standard deviation (Std dev), standard error (Std err), lower and upper limits for 95% confidence intervals (CI) for each gene by temperature (temp) treatment and month. Months are coded as November (1), December (2), January (3), February (4), March (5), April (6), May (7), and June (8).

Gene	Temp	Month	Mean copy num	Std dev	Std err	95% CI Lower	Upper
dual specificity mitogen-activated protein kinase kinase dSOR1 (ERK)	Constant	1	93.63	3.92	1.96	87.40	99.86
		2	90.48	24.87	12.43	50.91	130.05
		3	71.28	15.21	7.60	47.09	95.48
		4	87.47	26.93	13.46	44.63	130.31
		5	61.79	6.42	3.21	51.58	72.00
		6	67.52	12.89	6.45	47.01	88.04
		7	63.60	11.48	5.74	45.34	81.87
		8	56.75	3.12	1.56	51.78	61.71
forkhead box protein O	Fluctuating	1	373.83	125.34	62.67	174.39	573.27
		2	475.64	129.73	64.86	269.21	682.07
		3	465.35	43.31	21.65	396.44	534.26
		4	537.67	135.92	67.96	321.39	753.96
		5	494.22	97.61	48.81	338.90	649.55
		6	302.06	61.43	30.71	204.31	399.80
		7	279.35	55.97	27.99	190.29	368.41
		8	193.14	43.40	21.70	124.07	262.20
	Constant	1	556.11	49.96	24.98	476.60	635.61
		2	570.33	119.37	59.68	380.39	760.27
		3	558.48	98.24	49.12	402.16	714.80
		4	649.35	217.70	108.85	302.94	995.76
		5	572.23	141.43	70.72	347.17	797.28
		6	595.28	45.01	22.51	523.66	666.90
		7	718.00	212.13	106.07	380.45	1055.54
		8	617.50	46.61	23.30	543.34	691.66
insulin receptor-like	Fluctuating	1	351.50	246.00	123.00	-39.94	742.95
		2	525.76	287.15	143.57	68.84	982.67
		3	239.85	167.21	83.61	-26.22	505.92
		4	666.46	389.13	194.57	47.27	1285.65
		5	649.19	307.15	153.58	160.45	1137.94
		6	335.70	95.13	47.56	184.33	487.06
		7	109.16	121.12	60.56	-83.57	301.88
		8	108.52	135.88	67.94	-107.70	324.74

Table 7. Summary statistics for genes with significantly different expression levels as determined by nCounter analysis (continued). Summary statistics for each gene include mean copy number (Mean copy num), standard deviation (Std dev), standard error (Std err), lower and upper limits for 95% confidence intervals (CI) for each gene by temperature (temp) treatment and month. Months are coded as November (1), December (2), January (3), February (4), March (5), April (6), May (7), and June (8).

Gene	Temp	Month	Mean copy num	Std dev	Std err	95% CI Lower	Upper
insulin receptor-like	Constant	1	445.02	333.56	166.78	-85.74	975.78
		2	420.05	675.08	337.54	-654.15	1494.25
		3	493.95	188.15	94.07	194.56	793.34
		4	847.99	256.95	128.47	439.13	1256.85
		5	907.93	412.09	206.05	252.20	1563.67
		6	832.84	333.42	166.71	302.29	1363.39
		7	350.15	311.64	155.82	-145.74	846.04
		8	830.53	256.57	128.29	422.27	1238.80
insulin-like growth factor I	Fluctuating	1	6.18	3.28	1.64	0.97	11.39
		2	6.95	2.71	1.35	2.64	11.26
		3	6.84	4.17	2.08	0.21	13.48
		4	7.27	7.07	3.54	-3.99	18.52
		5	13.25	16.49	8.25	-12.99	39.49
		6	33.36	24.42	12.21	-5.50	72.21
		7	13.00	7.66	3.83	0.82	25.18
		8	14.70	10.46	5.23	-1.95	31.34
	Constant	1	7.47	3.17	1.59	2.42	12.52
		2	9.47	4.23	2.12	2.73	16.20
		3	10.70	0.95	0.47	9.20	12.21
		4	6.96	4.85	2.42	-0.76	14.68
		5	2.33	1.45	0.72	0.03	4.63
		6	5.66	3.11	1.55	0.72	10.61
		7	5.99	4.94	2.47	-1.87	13.85
		8	4.88	2.67	1.33	0.63	9.12
insulin-like receptor	Fluctuating	1	158.50	18.49	9.25	129.07	187.93
		2	190.27	31.71	15.85	139.81	240.72
		3	135.65	21.74	10.87	101.06	170.24
		4	128.74	44.68	22.34	57.65	199.84
		5	137.25	37.82	18.91	77.07	197.42
		6	119.89	13.95	6.97	97.70	142.08
		7	53.20	5.89	2.95	43.83	62.58
		8	47.59	16.05	8.02	22.05	73.12

Table 7. Summary statistics for genes with significantly different expression levels as determined by nCounter analysis (continued). Summary statistics for each gene include mean copy number (Mean copy num), standard deviation (Std dev), standard error (Std err), lower and upper limits for 95% confidence intervals (CI) for each gene by temperature (temp) treatment and month. Months are coded as November (1), December (2), January (3), February (4), March (5), April (6), May (7), and June (8).

Gene	Temp	Month	Mean copy num	Std dev	Std err	95% CI Lower	Upper
insulin-like receptor	Constant	1	199.78	42.23	21.12	132.57	266.99
		2	184.71	13.49	6.74	163.24	206.17
		3	157.90	23.00	11.50	121.30	194.49
		4	156.21	52.95	26.48	71.94	240.47
		5	158.82	27.39	13.70	115.22	202.41
		6	130.37	8.55	4.28	116.76	143.97
		7	121.87	29.15	14.58	75.47	168.26
		8	138.46	32.66	16.33	86.50	190.42
mitogen-activated protein kinase 1 (MAPK1)	Fluctuating	1	53.59	18.00	9.00	24.94	82.23
		2	47.22	25.64	12.82	6.42	88.03
		3	36.01	11.77	5.89	17.28	54.74
		4	39.18	11.91	5.96	20.22	58.13
		5	32.86	5.13	2.56	24.70	41.01
		6	33.86	20.55	10.27	1.166	66.55
		7	36.25	17.60	8.80	8.25	64.24
		8	36.12	15.73	7.87	11.07	61.16
	Constant	1	61.84	24.80	12.40	22.37	101.30
		2	49.11	7.42	3.71	37.30	60.91
		3	44.26	3.59	1.80	38.55	49.98
		4	31.65	16.81	8.41	4.89	58.40
		5	26.08	16.54	8.27	-0.23	52.39
		6	28.55	15.30	7.65	4.19	52.90
		7	29.16	15.30	7.65	4.81	53.50
		8	16.01	9.95	4.97	0.17	31.83
mitogen-activated protein kinase 14B-like (MAPK14B)	Fluctuating	1	246.75	6.04	3.02	237.13	256.36
		2	227.80	23.96	11.98	189.66	265.93
		3	221.39	23.25	11.63	184.39	258.39
		4	219.36	19.38	9.69	188.52	250.20
		5	232.88	26.18	13.09	191.23	274.53
		6	226.56	13.17	6.58	205.61	247.52
		7	228.33	72.25	36.13	113.37	343.30
		8	135.60	33.03	16.51	83.05	188.16

Table 7. Summary statistics for genes with significantly different expression levels as determined by nCounter analysis (continued). Summary statistics for each gene include mean copy number (Mean copy num), standard deviation (Std dev), standard error (Std err), lower and upper limits for 95% confidence intervals (CI) for each gene by temperature (temp) treatment and month. Months are coded as November (1), December (2), January (3), February (4), March (5), April (6), May (7), and June (8).

Gene	Temp	Month	Mean copy num	Std dev	Std err	95% CI Lower	Upper
mitogen-activated protein kinase 14B-like (MAPK14B)	Constant	1	213.95	10.46	5.23	197.30	230.60
		2	225.74	28.49	14.24	180.41	271.07
		3	219.16	4.44	2.22	212.09	226.22
		4	265.50	73.56	36.78	148.46	382.54
		5	239.64	29.10	14.55	193.34	285.93
		6	204.56	43.47	21.73	135.39	273.73
		7	213.51	10.82	5.41	196.29	230.73
		8	230.88	23.92	11.96	192.81	268.94
phosphatidylinositol 3-kinase catalytic subunit type 3	Fluctuating	1	67.11	17.60	8.80	39.09	95.12
		2	57.89	9.66	4.83	42.52	73.26
		3	65.71	6.86	3.43	54.79	76.62
		4	72.28	7.23	3.62	60.77	83.78
		5	98.12	12.24	6.12	78.64	117.59
		6	98.53	10.51	5.26	81.80	115.26
		7	86.85	26.71	13.35	44.35	129.35
		8	61.99	22.78	11.39	25.75	98.23
	Constant	1	87.79	13.95	6.97	65.60	109.98
		2	76.55	15.11	7.55	52.51	100.59
		3	82.13	14.49	7.25	59.07	105.19
		4	94.05	15.91	7.96	68.73	119.37
		5	86.45	26.81	13.40	43.80	129.11
		6	93.34	20.86	10.43	60.15	126.52
		7	117.77	33.45	16.72	64.55	170.98
		8	67.43	11.06	5.53	49.84	85.02
phosphatidylinositol 3-kinase regulatory subunit alpha (p85)	Fluctuating	1	236.86	40.22	20.11	172.87	300.86
		2	301.75	31.06	15.53	252.33	351.17
		3	318.61	57.67	28.83	226.85	410.37
		4	275.30	28.47	14.23	230.00	320.60
		5	354.64	52.59	26.30	270.95	438.33
		6	296.00	37.30	18.65	236.64	355.35
		7	107.52	45.21	22.61	35.57	179.47
		8	132.53	34.01	17.01	78.41	186.66

Table 7. Summary statistics for genes with significantly different expression levels as determined by nCounter analysis (continued). Summary statistics for each gene include mean copy number (Mean copy num), standard deviation (Std dev), standard error (Std err), lower and upper limits for 95% confidence intervals (CI) for each gene by temperature (temp) treatment and month. Months are coded as November (1), December (2), January (3), February (4), March (5), April (6), May (7), and June (8).

Gene	Temp	Month	Mean copy num	Std dev	Std err	95% CI Lower	Upper
phosphatidylinositol 3-kinase regulatory subunit alpha (p85)	Constant	1	206.86	17.15	8.58	179.57	234.16
		2	271.87	36.47	18.24	213.83	329.90
		3	386.32	87.98	43.99	246.32	526.32
		4	348.70	134.15	67.07	135.24	562.16
		5	396.81	52.21	26.10	313.73	479.89
		6	404.55	36.16	18.08	347.00	462.09
		7	353.57	57.45	28.72	262.17	444.98
		8	418.08	81.07	40.53	289.08	547.08
phosphatidylinositol 3/4/5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase (PTEN)	Fluctuating	1	50.76	5.11	2.56	42.61	58.90
		2	49.64	7.74	3.87	37.31	61.96
		3	42.53	3.80	1.90	36.48	48.58
		4	35.71	11.15	5.57	17.97	53.44
		5	41.04	9.82	4.91	25.41	56.67
		6	45.81	15.00	7.50	21.94	69.67
		7	63.90	24.46	12.23	24.98	102.82
		8	50.54	19.76	9.88	19.10	81.99
	Constant	1	60.08	9.85	4.93	44.40	75.75
		2	53.80	12.92	6.46	33.23	74.37
		3	40.63	13.54	6.77	19.08	62.18
		4	49.88	15.32	7.66	25.51	74.25
		5	40.76	14.42	7.21	17.81	63.71
		6	37.98	10.70	5.35	20.95	55.01
		7	36.75	6.74	3.37	26.03	47.47
		8	42.65	7.37	3.68	30.93	54.37
ras-like protein 2	Fluctuating	1	140.86	8.10	4.05	127.97	153.75
		2	151.53	8.65	4.33	137.76	165.30
		3	153.98	14.63	7.32	130.70	177.26
		4	175.18	30.53	15.27	126.59	223.76
		5	190.97	34.72	17.36	135.73	246.21
		6	192.70	25.31	12.66	152.42	232.97
		7	183.59	45.03	22.52	111.93	255.25
		8	153.61	34.11	17.06	99.33	207.90

Table 7. Summary statistics for genes with significantly different expression levels as determined by nCounter analysis (continued). Summary statistics for each gene include mean copy number (Mean copy num), standard deviation (Std dev), standard error (Std err), lower and upper limits for 95% confidence intervals (CI) for each gene by temperature (temp) treatment and month. Months are coded as November (1), December (2), January (3), February (4), March (5), April (6), May (7), and June (8).

Gene	Temp	Month	Mean copy num	Std dev	Std err	95% CI Lower	Upper
ras-like protein 2	Constant	1	193.14	24.36	12.18	154.37	231.90
		2	201.98	34.40	17.20	147.24	256.72
		3	200.59	35.26	17.63	144.48	256.70
		4	191.02	40.52	20.26	126.55	255.50
		5	212.90	48.85	24.43	135.16	290.623
		6	158.40	19.60	9.80	127.21	189.58
		7	164.93	17.04	8.52	137.81	192.05
		8	176.95	22.60	11.30	141.00	212.90

Effects of temperature on IIS

Next, I looked at whether temperature affected gene expression by comparing monthly expression levels between temperature treatment groups. My results showed that *cyclin D*, *cyclin E*, *cyclin G*, *FOXO*, *GAB2*, *insulin-like receptor*, *insulin receptor-like*, *MAPK14B*, *PI3K* regulatory subunit, *RAS2*, and *samui* all varied between temperature treatments at a given month or over time within a treatment (Table 6).

The slope for *cyclin D* expression levels in the fluctuating treatment group was significantly lower than that of the constant temperature group (Table 6; slope: estimate = -6.59, DF = 64, $p < 0.01$) In November, *RAS2* was significantly higher in the constant treatment group (Fig. 23E; intercept: estimate = -65.90, DF = 30.61, $p < 0.0001$). Temperature treatment significantly affected expression levels of *cyclins E* and *G* during the middle months of overwintering. In January, bees in fluctuating temperatures had approximately 30% lower mean copy number for *cyclin G* compared to bees in constant temperature (Constant: 348.95 ±39.18; Fluctuating: 240.26±21.02). Bees in the fluctuating temperature group showed overall lower

expression of *cyclin E*, but was significantly lower than the constant temperature group from January through June (Fig. 21B; slope: estimate = -22.54 DF = 63.60, p <0.001).

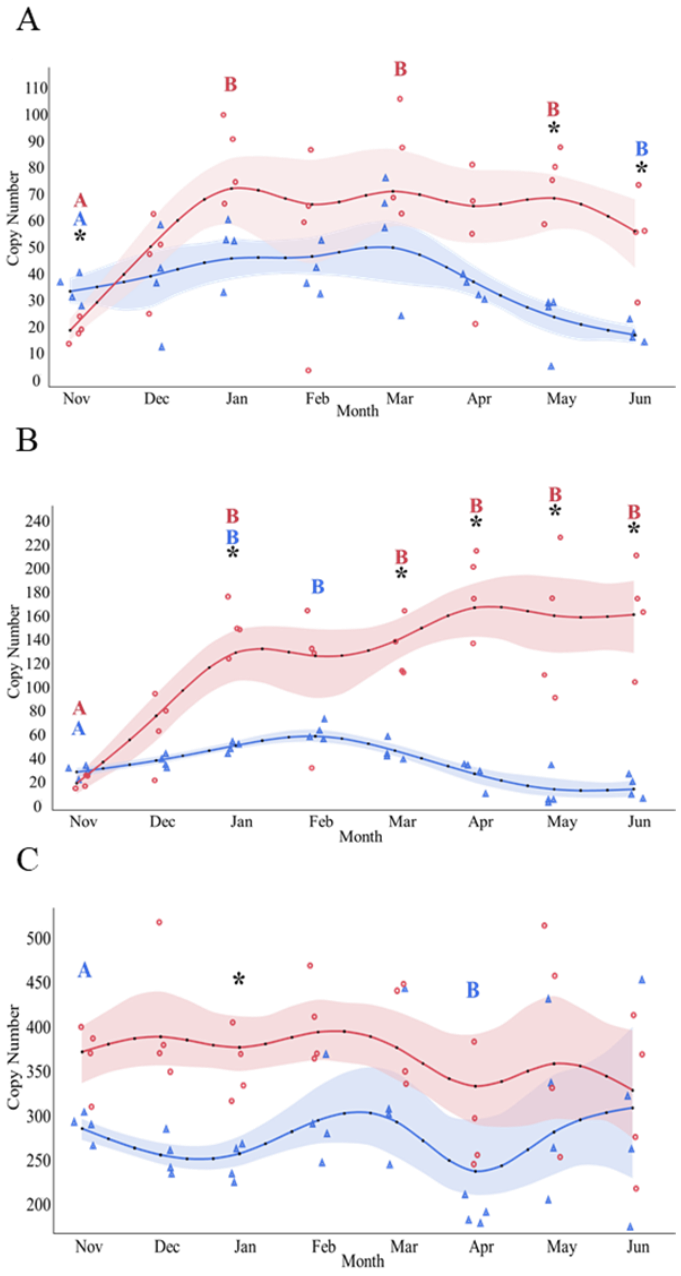


Figure 21. Expression of cyclin genes. Cell cycle genes, A) *cyclin D*, B) *cyclin E*, and C) *cyclin G*, were significantly different by location and over time. Fluctuating temperatures samples are shown by blue triangles and constant temperature samples are shown by red circles. Months with different letters are significantly different. Letter colors correspond to temperature treatment. 95% confidence intervals are shown in shaded areas. Asterisks indicate significant differences between temperature treatments at a given month.

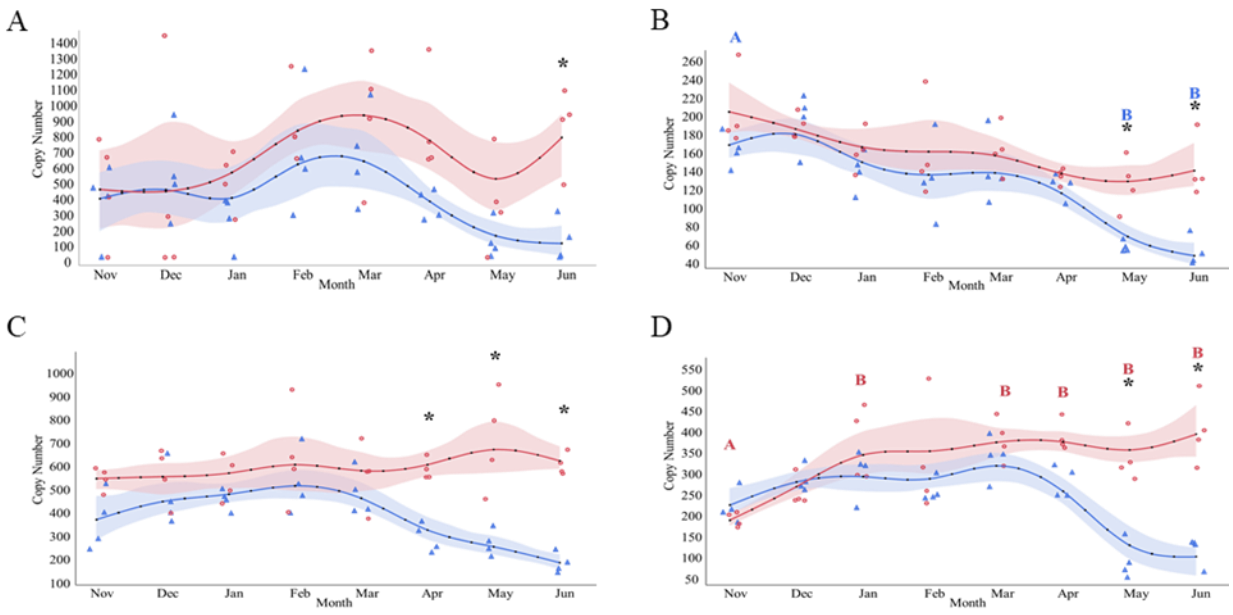


Figure 22. Expression of genes in the insulin pathway. Genes in the insulin signaling pathway that showed significant differences included A) *insulin receptor-like* B) *insulin-like receptor*, C) *FOXO*, and D) *PI3K* regulatory subunit. Fluctuating temperatures samples are shown by blue triangles and constant temperature samples are shown by red circles. Months with different letters are significantly different. Letter colors correspond to temperature treatments. 95% confidence intervals are shown in shaded areas. Asterisks indicate significant differences between temperature treatments at a given month.

During the end of the overwintering period, April, May, and June, several genes showed significant differences between temperature treatments. Results showed fluctuating temperature groups being significantly lower than the constant temperature groups for *FOXO* (Fig. 21C; slope: estimate = -47.39, DF = 64, p <0.001), *GAB2* (Fig. 23B; slope: estimate = -23.23, DF = 62.86, p <0.001), *samui* (Fig. 23F; slope: estimate = -170.30, DF = 64, p = 0.01), *cyclin D* (Fig. 21A; slope: estimate = -6.59, DF = 64, p <0.01), *insulin-like receptor* (Fig. 22B; slope: estimate = -8.06, DF = 64, p = 0.02), *insulin receptor-like* (Fig. 22A; slope: estimate = -82.61, DF = 64, p = 0.03), *MAPK14B* (Fig. 23D; slope: estimate = -9.67, DF = 56.16, p = 0.01), and *PI3K* regulatory subunit (Fig. 22D; slope: estimate = -41.27, DF = 63.90, p <0.001).

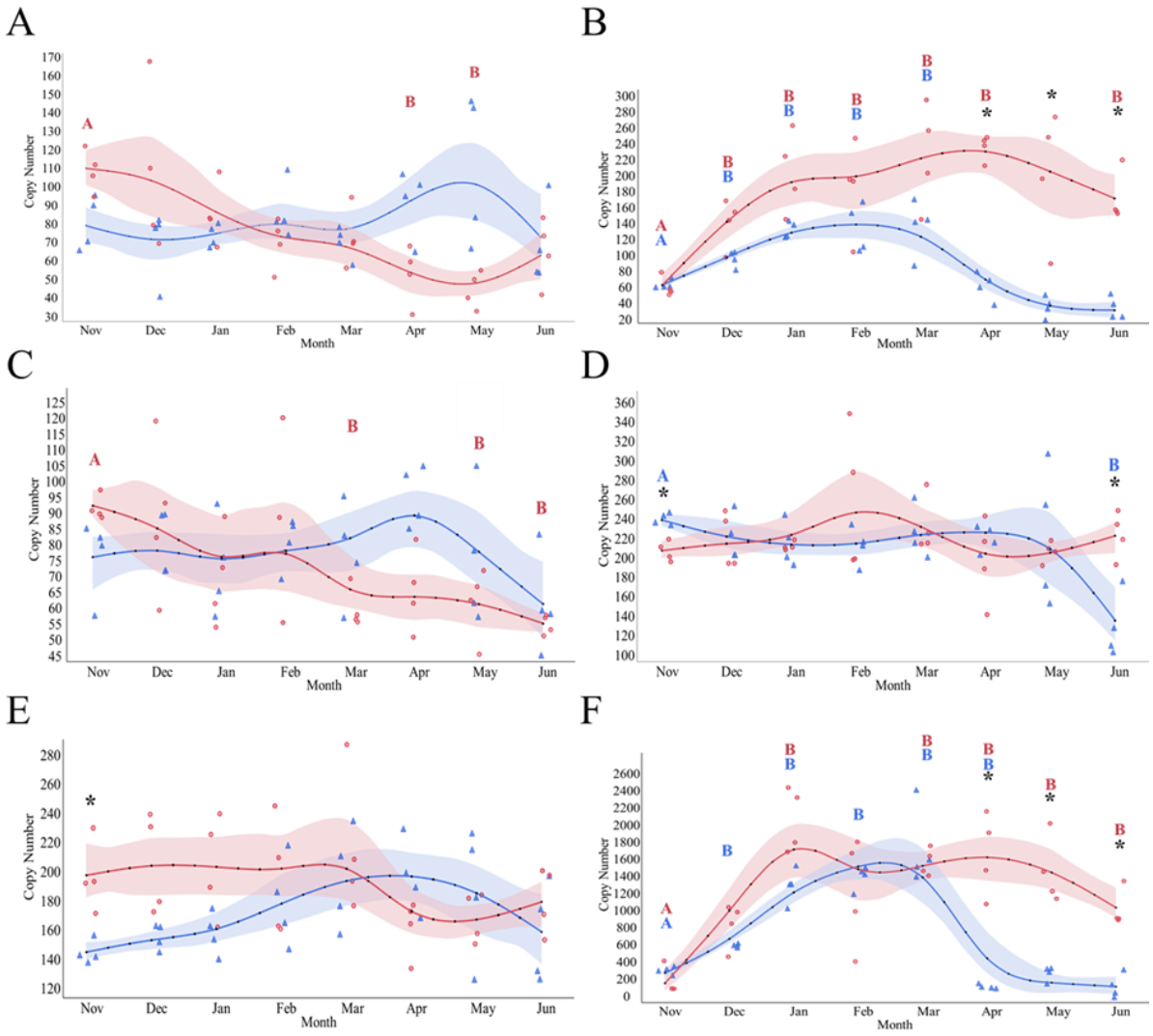
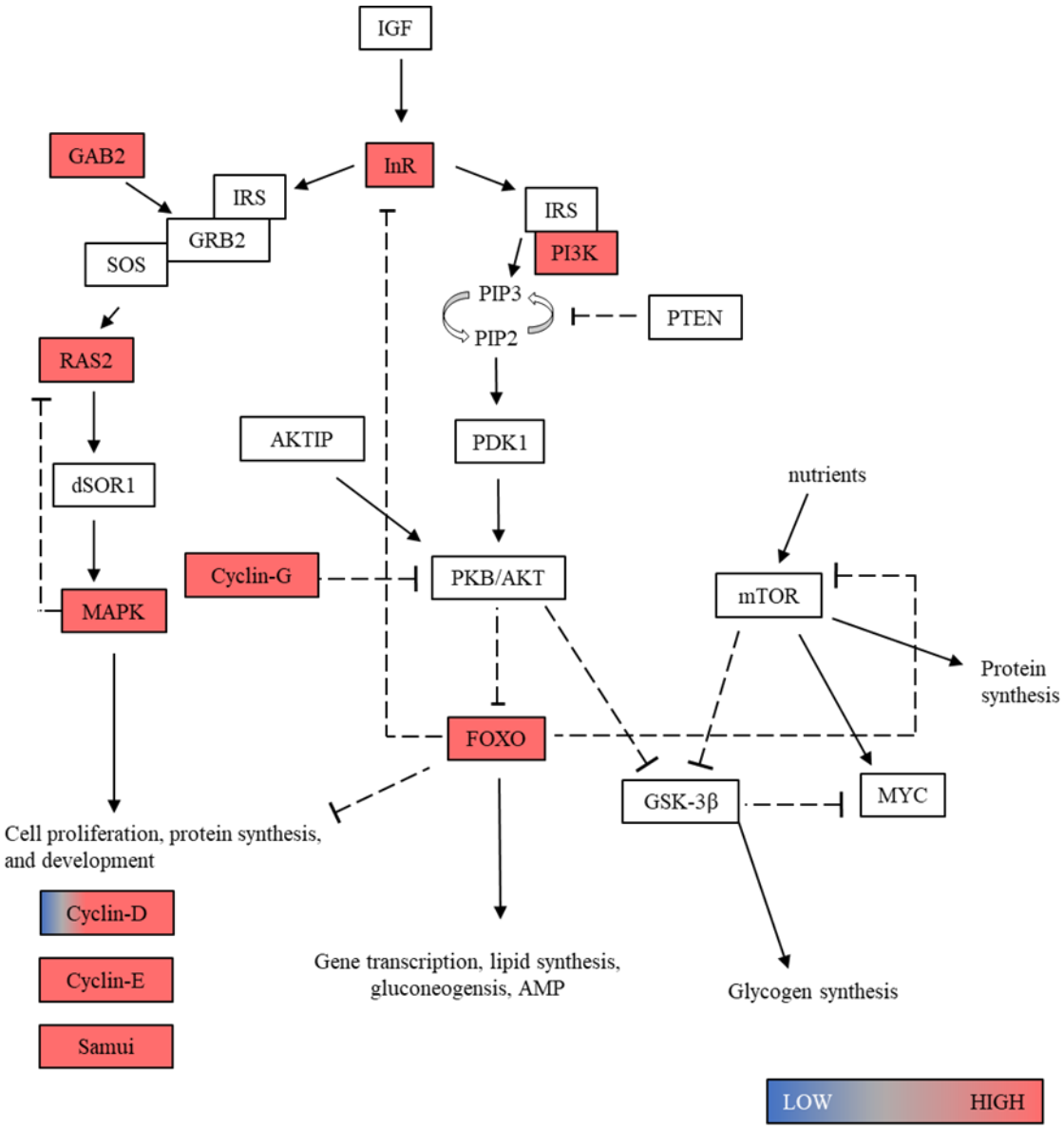


Figure 23. Expression of regulatory genes. Regulatory genes such as A) *AKT-interacting protein*, B) *GAB2*, C) *ERK*, D) *MAPK14B*, E) *RAS2*, and F) *samui* were significantly different by location and over time. Fluctuating temperatures samples are shown by blue triangles and constant temperature samples are shown by red circles. Months with different letters are significantly different. Letter colors correspond to temperature treatment. 95% confidence intervals are shown in shaded areas. Asterisks indicate significant differences between temperature treatments at a given month.

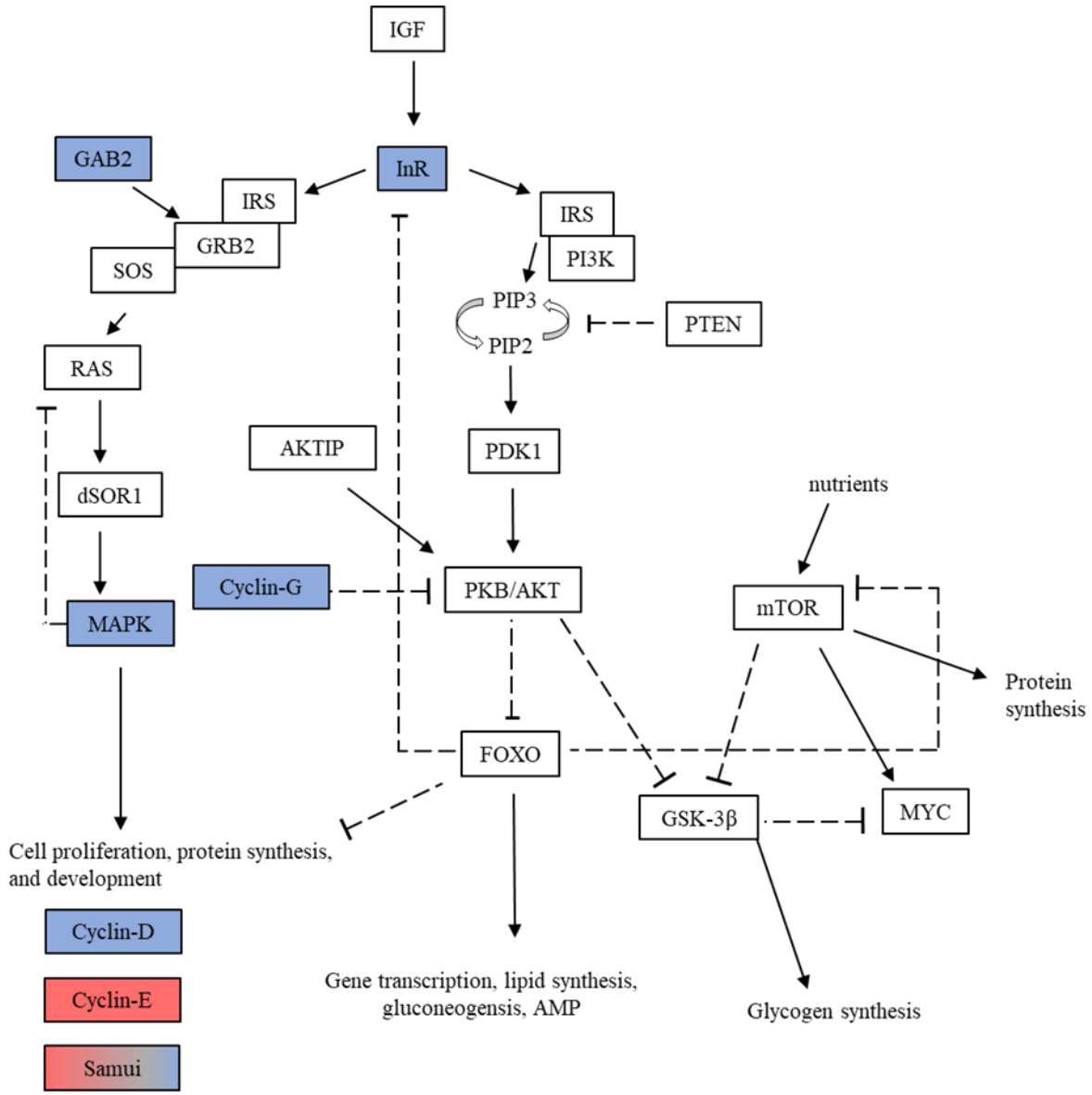
Discussion

This study is the first to use this technique in a Hymenopteran and to investigate IIS signaling in an overwintering solitary bee. Overall my results showed that a few key targets of the insulin signaling pathway along with growth regulators change during overwintering, suggesting that only cell cycle regulators, and not the insulin signaling pathway as a whole, are changing across the phases of diapause. Of the genes that were significantly different over time, only 3 were targets of the insulin signaling pathway, not supporting my hypothesis. The other genes, cyclins and samui, responded over time as expected based on their roles in regulating development. My second question was whether temperature impacted IIS gene expression, so I compared gene expression levels between temperature treatments at each month for a given gene. When looking at the effects of temperature on gene expression, I observed significantly more differences in expression of target genes of the insulin signaling pathway, supporting the hypothesis that insulin signaling may be involved in regulating energy reserves needed in response to fluctuating temperatures. The results showed that, again all three *cyclins* (D, E, G), *FOXO*, *GAB2*, *insulin-like receptor* and *insulin-receptor like*, *MAPK14B*, *PI3K* regulatory subunit, and *samui* varied between constant and fluctuating temperature groups at several time points (Fig. 24). This was expected as insects go through the different developmental phases of diapause. Overall my findings indicate that insulin signaling is either suppressed or downregulated during overwintering in *M. rotundata*, supporting previous studies on insulin signaling in diapause regulation in other insects.



MONTHS OF SIGNIFICANT DIFFERENCES				
InR: 8	**PI3K Reg:** 5,6	**FOXO:** 6-8	**GAB2:** 6-8	**RAS2:** 1
MAPK14B: 8	**Cyclin D:** 1,7, 8	**Cyclin E:** 1, 3, 5-8	**Cyclin G:** 3	**Samui:** 7, 8

Figure 24. Gene expression of IIS pathway between temperature treatments. Gene expression relative to that of the fluctuating temperature treatment group is shown as either lower (blue) or higher (red) for the constant temperature treatment group. The months that these differences were seen are indicated in the table. Months include November (1), December (2), January (3), February (4), March (5), April (6), May (7), and June (8).



MONTHS OF SIGNIFICANT DIFFERENCES				
InR: 7,8	**Cyclin D:** 8	**Cyclin E:** 3,4	**Cyclin G:** 6	**GAB2:** 2-5
MAPK14B: 8	**InR:** 7,8	**Samui:** 2-6		

Figure. 25. Gene expression of IIS pathway over time during overwintering months. Genes that were significantly different relative to November are shown in blue (lower) or red (higher). The months that these differences were seen are indicated in the table. Months include November (1), December (2), January (3), February (4), March (5), April (6), May (7), and June (8).

In addition, I was able to compare results from both techniques since several of the same samples and the same genes were used in both. Although qPCR only showed a difference in gene expression for 3 genes (Fig. 18), nCounter results detected 17 genes that differed significantly with temperature (Table 6). When looking at how insulin signaling changed over time, I observed differences in the *insulin-like receptor*, *GAB2*, *MAPK14B*, all three cell cycle genes (*cyclins D, E, G*), and *samui* (Fig. 25). The finding that qPCR showed fewer differences than NanoString is possibly due to a large coefficient of variation and small sample size in the qPCR study (Table 5). Another reason for the mismatch in results between techniques is likely because many of the differences found with NanoString occurred in April, May, and June, and samples from these months were not used for qPCR. For the months that were tested using both techniques (Nov, Dec, Jan, Mar), results were consistent between the two methods. My samples originated from a previous Illumina study (Yocum et al., 2018). When comparing results from Yocum et. al (2018) to the current results, genes like *samui* and *insulin receptor-like* behaved similarly over time. Interestingly, many of the differences between the temperature treatment groups seen in the Illumina study occur in May, similar to what was found using NanoString.

Overwintering consists of different phases of diapause (initiation, maintenance, termination) and post-diapause quiescence, and thus I expected the gene expression levels to reflect these different stages (Koštál, 2006). Hierarchical clustering of nCounter results showed separation of gene expression into three clusters that reflect the phases of diapause: 1) November and December, 2) January through March, and 3) April through June (Fig. 19). Most differences between temperature treatments occurred during April, May, and June, the months when the transition from post-diapause quiescence to direct development would occur. Direct development is initiated when temperatures are greater than 18˚C (Undurraga and Stephen, 1980). Average

temperatures increased during these three months from April (7.4˚C), to May (13˚C) and June (22˚C) (Fig. S1;Yocum et al., 2018), suggesting that bees in the constant temperature group did not receive appropriate thermal cues to progress to direct development. Ecophysiological phases of diapause have been studied in the drosophilid fly, *Chymomyza costata*, using transcriptomic data (Kostal et al., 2017). Different developmental phases of diapause were distinguished by identifying characteristic patterns of gene expression for each phase. In diapause-destined larvae, gene expression profiles showed inhibition of developmental hormone signaling and as a result, down-regulation of transcriptional factors leading to cell cycle arrest (Kostal et al., 2017). Cell cycle regulators like the cyclins and transcription factors such as *samui* were differentially expressed over time, similar to results in the current study. Although my study was not focused on searching for genes involved with diapause regulation, these results may be a starting point for predicting the diapause developmental stages in *M. rotundata*.

Our results of no change in IIS pathway gene expression over time (Fig. 22) supports the idea that continued suppression of IIS over time is important for diapause shown by previous reproductive diapause studies. For both *Culex pipiens* and *D. melanogaster*, insulin signaling has been strongly tied to regulating reproductive diapause (Kubrak et al., 2014; Kubrak et al., 2016; Nässel et al., 2013; Schiesari et al., 2016; Sharma et al., 2019; Sim and Denlinger, 2009; Zhang et al., 2017). Reproductive diapause in the mosquito *Cx. pipiens* has been shown to be regulated by the downstream IIS target, *FOXO* (Sim and Denlinger, 2007, 2008; Sim et al., 2015). Through RNAi knockdown of the insulin receptor, Sim et. al (2007) were able to produce a diapause phenotype as shown by an arrest in ovarian development in *Cx. pipiens*. This supported what was previously found for reproductive diapause in the fruit fly, *D. melanogaster*. The gene for *phosphatidylinositol 3-kinase* (PI3K) in *D. melanogaster*, *Dp110*, was shown to be a key

regulator of diapause in response to environmental stress (Williams et al., 2006). Over the course of nine weeks of diapause, *D. melanogaster* exhibited changes in several dILP mRNA levels but not in insulin receptor mRNA (Kubrak et al., 2014). This suggests that in response to unfavorable conditions like those during diapause, flies alter their metabolic homeostasis, which in turn alters insulin signaling. This allows for required resources, such as circulating sugars, to be allocated for survival (Kubrak et al., 2014). Additionally, mutant strains of *D. melanogaster* that were insulin-deficient showed more ovarian development arrest, indicating a strong connection between diapause induction and reduced insulin signaling (Kubrak et al., 2014). Few studies have investigated the complex relationship between insulin signaling and diapause regulation in insects that overwinter at different developmental stages. For instance, Kostal et al. (2017) investigated diapause regulation in the drosophilid fruitfly, *C. costata*, which enter diapause in the third larval instar. Although this study did not specifically focus on insulin signaling, genetic profiles of the different phases of diapause development in *C. costata* larvae showed very few differences in IIS/TOR pathway signaling. In the current study in prepupae, I also found no differences in target genes of the IIS pathway, such as *PI3K*, *FOXO*, and *insulin receptors* (Fig. 22) over time in the fluctuating temperature treatment group. To elucidate the mechanism(s) behind diapause regulation more diapause and insulin signaling studies at different developmental stages are needed.

Evidence from previous studies show that diapause may be regulated by several mechanisms. The moth *Pieris brassicae* diapauses in the next developmental stage, pupae, and were stimulated to resume adult development when injected with bovine insulin, suggesting a role of IIS in diapause regulation (Arpagaus, 1987). In the cotton bollworm *Helicoverpa armigera*, which diapauses as a pupa, reactive oxygen species extend life span by using

downstream components of the insulin signaling pathway, such as *FOXO*, to induce physiological responses that promote resistance to low temperature, oxidative stress, and pathogenic infections (Zhang et al., 2017). Life span extension, a characteristic of diapause, is regulated in response to levels of oxidative stress in *D. melanogaster* (Wang et al., 2003) and *C. elegans* (Schulz et al., 2007; Yang and Hekimi, 2010). The results from these studies collectively suggest insulin signaling pathway plays an important role in diapause regulation, regardless of the stage in which the insect diapauses.

The IIS pathway is clearly involved in diapause regulation, but how this pathway is utilized seems to vary. A recent meta-analysis of diapause transcriptomic data sets compared how diapause responses varied across species, and throughout ecophysiological phases of diapause development (Ragland and Keep, 2017). With data sets representing three orders (Diptera, Lepidoptera, and Hymenoptera) and various diapause stages from larva to adult, results showed that a core set of differentially regulated genes included targets of the insulin signaling pathway (Ragland and Keep, 2017). However, these genes interacted with several other pathways such as JNK and TOR to modulate insulin signaling (Ragland and Keep, 2017), possibly indicating plasticity in how the insulin signaling pathway is regulated during diapause since both pathways interconnect with IIS at different points. With insufficient studies covering more species and diapause stages, much remains to be understood about the regulatory role of insulin signaling in diapause.

Although previous studies to determine diapause regulation mechanisms in solitary bees have been inconclusive in elucidating a complete mechanism (Forrest et al., 2019; Kemp and Bosch, 2001), the role of temperature has been shown to be important (Kemp and Bosch, 2001; Tepedino and Parker, 1986; Yocum et al., 2018; Yocum et al., 2005, 2006). Several

transcriptome studies have scratched the surface on diapause regulation in *M. rotundata* (Torson et al., 2015; Yocum et al., 2018; Yocum et al., 2015). However, these studies only provide a macro-scale view of what is occurring. My results for bees that were overwintered in the field were expected, however, those kept in a constant temperature showed drastically different outcomes. Previous studies have also shown differential effects between constant and fluctuating temperatures in *M. rotundata* (Colinet et al., 2018; Rinehart et al., 2016; Rinehart et al., 2013; Torson et al., 2015; Yocum et al., 2018; Yocum et al., 2005, 2006; Yocum et al., 2012; Yocum et al., 2010). Because insects are ectotherms, their metabolic rate is directly correlated with temperature. If insulin signaling is indeed regulating energy stores, one would expect insulin signaling to also vary with temperature. In solitary bees exposed to a rapid pulse of high temperature, metabolic rates increase (Yocum et al., 2011) and genes in metabolic pathways are upregulated (Torson et al., 2015). My study showed similar results with several genes of the insulin signaling pathway, such as *PI3K*, *FOXO*, and *insulin receptors* (Fig. 22), significantly different between treatment groups, indicating a response of IIS to temperature.

Information about the environmental requirements for diapause, such as temperature, humidity, and photoperiod, has helped establish optimal storage conditions for commercially managed insects (Bloch et al., 2010; Goldsmith et al., 2005; MacKenzie et al., 1997; Pitts-Singer and James, 2009; Rinehart et al., 2011). Although these questions about diapause are being studied in a variety of insects, it is still vastly understudied in agriculturally important pollinators. The alfalfa leafcutting bee is the most commercially-managed solitary pollinator and has been unsuccessful in sustaining colonies in the US. With a wide range of environments due to high commercial management, it is unclear how environmental factors, such as temperature, impact diapause regulation and colony sustainability in *M. rotundata*. This study adds to the

growing knowledge of *M. rotundata* physiology. As a non-model organism, many molecular tools are not yet optimized for answering these questions in *M. rotundata*. It is unclear whether my results are true for other solitary or social bees, therefore further studies are needing in other solitary bees.

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CHAPTER 5. CONCLUSION

Insects are the largest and most diverse group of animals, but less than a third of them have been identified (Engel and Grimaldi, 2004). Of those that have been identified, much about their physiology remains unknown (Engel and Grimaldi, 2004). For example, how energy reserves are regulated is unclear , especially when energy reserves are limited. With several conserved pathways between vertebrates and invertebrates, it is likely that a network of pathways are involved in regulation of energy stores, what pathways and how they are connected have yet to be clearly elucidated. Additionally, how those networks change in response to different energy demands, such as immune challenges or changes in metabolism, is poorly understood.

There are several points during the insect life cycle that are non-feeding: metamorphosis, pupation, overwintering. My dissertation research provided insight into the regulation of physiological processes during non-feeding periods in two agriculturally relevant insects, *Manduca sexta* and *Megachile rotundata*. My study is the first to investigate the role of insulin signaling in diapause regulation in a solitary bee and to use the nCounter technique with an insect other than *Drosophila melanogaster*. It is important to know what physiological and biochemical changes are occurring during non-feeding periods to better understand how those changes impact insect physiology in the short and long term. Effects of short-term non-feeding periods can help us answer some of these developmental stage-specific questions like within a larval instar. Longer non-feeding periods like diapause can have even broader impacts, providing critical knowledge of the physiology across several developmental stages. All these questions are still unanswered, particularly for non-model insects. My research filled the gap on different physiological changes in non-feeding periods like nutrient regulation, immunity, and overwintering.

Chapter 2 focused on the regulation of a specific type of stored energy reserve, lipids. Results from this chapter suggested that high-fat diet-fed *Manduca sexta* larvae consumed food until reaching a maximum lipid storage capacity. I proposed that a feedback mechanism disrupts feeding and causes caterpillars to switch from lipid storage to excretion. This has been the only study to show high-fat diet-induced anorexia in an insect, contradicting the current knowledge on the effects of high-fat diets in *Manduca sexta*. Although no longer a major crop pest, knowledge of nutrient thresholds in *M. sexta* would still provide insight for potential pesticide targets for control of other crop pests. Other larval Lepidopterans like the codling moth (*Cydia pomonella*), the false codling moth (*Thaumatotibia lecucotreta*), and the pink bollworm (*Pectinophora gossypiella*) are still major pests of stored grains and food crops. As several of these crop pests develop insecticide resistance, it is important researchers continue finding different approaches for pest control. The lipid threshold of Lepidopterans could be used as a form of pest control by utilizing this feedback mechanism to mimic high-fat diet-induced anorexia, and therefore decrease their feeding. It would be interesting to repeat this study in diapause-destined *M. sexta* larvae, which would require higher lipid storage, and may have a different lipid threshold as a result. Teasing apart any pathway to further investigate this question can be complicated when multiple signaling factors act simultaneously. These types of limitations in insect physiology studies may be addressed as new techniques becoming available and non-feeding periods can be further studied.

The non-feeding period in my following chapter was a cessation of feeding during an immune challenge. Results from Chapter 3 determined that insulin signaling does not directly regulate the immune response, sickness-induced anorexia (SIA). I hypothesized that insulin signaling regulated the cessation of feeding during SIA in *M. sexta*. However, insulin

manipulation had no effect on larval growth during SIA. To better understand what happens during SIA, I measured changes in gene expression and compared with controls. Immunity-related genes differed in expression more than insulin target genes, providing further support for the null hypothesis. The upregulation of Toll and a few insulin signaling pathway genes matched previous findings in starvation studies, suggesting that SIA yields a similar phenotype to starvation by using the same mechanism. Therefore, I proposed that Toll-mediated suppression of the insulin signaling pathway inhibits feeding during SIA. Although both starvation and SIA may lead to a Toll-mediated suppression of the insulin signaling pathway, it is possible that the initial triggers for both may differ.

I also found that SIA during larval development led to long-term effects on adult emergence, highlighting the importance of taking sufficient measurements post-treatment. Along with our previous study that showed immunity changes across development, especially in the 5th instar, these results suggest the importance of choosing the correct age to start treatments and the timing of measurements post-treatment. These factors are especially important for future *M. sexta* studies on insulin signaling since previous work has shown there are differences in developmental expression of IIS pathway members. Future studies should be considerate about what developmental stage they use in their studies, as well as the implications it can have on the remaining stage of an insect's life cycle.

As questions are answered in one insect, it cannot be assumed or applied to another insect if both were not conducted on the same age or developmental stage. This causes a limitation for the field of insect physiology because not all insects can be easily used at every developmental stage. An advantage to using 5th instar *M. sexta* is that both their large size and per volume equivalence to a small mouse allow for ample sample collections compared to smaller insect

species. Different developmental stages have different benefits for different insects, making it important that the correct developmental stage be used and that information only be applied when age is controlled between studies.

The importance of considering developmental stage also applies to diapause studies, where insects can overwinter at any point of a life cycle. *M. rotundata* overwinter as prepupae and have more development to undergo after diapause, making it difficult to apply knowledge from previous diapause studies. Therefore my research in Chapter 4 focused on determining 1) how insulin signaling varied throughout diapause and 2) if that variation changed in response to temperature in the alfalfa leafcutting bee *Megachile rotundata*. Overall my results showed that a few key targets of the insulin signaling pathway along with growth regulators change during overwintering, suggesting that only cell cycle regulators, such as cyclins, and not the insulin signaling pathway, change across the phases of diapause. When addressing my second question, I observed significantly more differences in expression of target genes of the insulin signaling pathway, indicating temperature influenced insulin signaling.

Studying diapause in *M. rotundata* is critical for two reasons. First, *M. rotundata* is an agriculturally important insect species. As polylectic bees, they pollinate a variety of crops and plants. Secondly, as a solitary bee, they are more representative of all Hymenopterans which are also mainly solitary. With effects of temperature treatment groups, constant temperatures like those used in commercial storage protocols, may cause different gene expression profiles and possibly different phenotypes in these bees. Farmers put bees into cold storage for overwintering at a constant temperature, which is already known to decrease survival of *M. rotundata* when used for long periods. This harmful storage protocol could be contributing to their reduced yield in the US.

More alarming is the fact that my findings show that different temperature treatments led to different genetic profiles of these bees, raising the question of how climate change will impact these bees. Genes in the insulin signaling pathway were higher in bees that were kept at a constant temperature during overwintering. One possible explanation is that without temperature cues, expression levels may have not decreased as much as if they were exposed to naturally fluctuating temperatures. If so, this can potentially be harmful if their metabolism, which is known to be regulated by insulin signaling, is not sufficiently decreased to allow for reserves to be slowly used for overwintering survival. Once these bees emerge from overwintering and resume development, shifts in seasons and increases in the frequency of spring cold snaps due to climate change, puts them at risk for cold damage. Another question my findings raise is how this will impact other bee species, especially eusocial bees like honeybees and wild bees. The insulin signaling pathway is known to regulate caste development and behavioral maturation in honeybees (Wheeler et al., 2006; Wolschin et al., 2011). However, if insulin signaling changes in response to temperature, it is unclear how the changes in temperature associated with climate change will impact insulin signaling in eusocial bees, and therefore their castes and hive survival. With both a lab and field setting, my study provides baseline knowledge of how insulin signaling changes during overwintering and in response to fluctuating temperature in *M. rotundata*. These baseline findings can help with conservation efforts for all solitary bees.

Whether focusing on a crop pest or an agriculturally relevant insect, insect physiology studies need to keep up with advances in technology just as biomedical research does. Classic molecular techniques range from Western blots to mass-spectrophotometry, but as technology advances, insect physiologists need to incorporate non-traditional approaches and novel techniques. There are several limitations in the classic techniques that can be overcome with new

technology. For example, the requirement of large amounts of cDNA samples and the cost associated with testing several genes of interest with qPCR can be overcome with the multiplex approach of nCounter that requires minimal amounts of RNA, not even cDNA. Reverse transcription reactions that create cDNA from RNA can be highly variable and prone to human error, leading to drastic differences even within the same samples (unpublished data). There was also no clear consensus in the field of how many control/reference genes one should use for proper normalization with qPCR. Since nCounter allows the user to decide the number of control genes they use, it is important to follow a standard of using at least three genes. Additionally, the equations to calculate fold-change in expression varied not only within insect physiology, but across all disciplines of biology. nCounter does have its own software for calculating differential expression, which may reduce this issue. Every technique has pros and cons, and unfortunately for many of them the price is a big con. A limitation with Chapter 4 is that I was limited to small sample size and fixed number of genes I could test with nCounter due to cost; however qPCR would have been even more expensive with its high reagent costs. In addition to cost, I'm sure over time there will be other limitations to using nCounter, but it will take repeated use to discover them.

Cost has been a huge limitation of another popular technique, RNAseq. However, as it becomes more affordable and therefore accessible to researchers, it has allowed for more genomic studies. An extensive RNAseq study in 2016 created a draft of the *M. sexta* genome and genetic profiles by tissue and developmental stage (Kanost et al., 2016). Per our request, NCBI reannotated the genome with information from subsequent studies in *M. sexta*. However, even with a draft genome and reannotation, the current genome for *M. sexta* still needs a lot of work before it can be as useful as the current *Drosophila* genome. There are several predicted variants

and potential isoforms of genes listed in the *M. sexta* genome. Clearly, further studies are needed to validate these predictions to better use these datasets to answer remaining questions in insect physiology. Due to the large number of variants that have not been validated, my custom nCounter probe set had to be designed to cover all potential isoforms, leaving the question of how individual genes change unanswered. Therefore, future studies will need to focus on investigating individual genes.

Another issue with annotations is the inconsistent use of nomenclature. Previous studies have used the terms ILP and bombyxins interchangeably, and the annotation of the *M. sexta* genome also includes both. Bombyxins were the first insect ILP discovered in the silkworm, *Bombyx mori*, hence many researchers continue to use the name bombyxins instead of ILP. These nomenclature issues make following the literature difficult. Using new technologies like nCounter will require trials, as with all techniques prior. But in order for the field of insect physiology to continue advancing, researchers have to take those first steps.

During my graduate career at NDSU, I also learned a vast number of life lessons and hope to share some of that knowledge with incoming and current students. First and foremost, make sure your research excites you. If you are as fortunate as I was and are able to design your own dissertation project, do what makes your little nerd heart pound. There will be long nights, early mornings, weekends, and holidays where you will be working and it will be even more difficult if you hate the topic or work. If you are not as fortunate and have to continue a previous student's project, then try to add your twist and flavor. This will still be YOUR project, so own it. When the moment comes, because it will, where you feel like you have lost interest or your passion for your topic, I highly recommend stepping back and reading other literature. You

surround your mind with so much knowledge of the same topic that it is very easy to lose sight of why you chose it in the first place.

Along with losing your *upmf* for your research topic, there will come a time where you no longer remember why you started graduate school. This is normal. You will question whether you will ever finish and whether you made the right choice even starting. Let me answer both of those for you: Yes. You will eventually finish but do not set yourself back by comparing your journey to another student's. Your projects will be different. Your funding source will be different. Your advisors and their mentoring styles will be different. Do not fixate on a finish line because you will run right past your other successes. As for whether starting graduate school was the right choice, I can only say yes it was the right choice because you made it. You have to learn to trust your own judgement, but only you can make that decision for yourself. There is no shame in leaving if you feel like you need to.

Shame is a topic I want to really discuss. As a Mexican-American daughter, I really struggled with balancing my cultural expectations and pursuing my PhD. The expectation for daughters is to be the caregiver for the parents, but also to start your own family. Pursuing a PhD is not normal for my culture and that was difficult to handle. It was hard to explain to family why I was not visiting as often, or when I would be able to see them next. Graduate school does not have defined hours or holidays and my family did not understand that. I missed several birthdays and family events. The Mexican culture is to always put family first and for the past six years I felt ashamed that it was not. I felt ashamed that I had to leave my family and community to work towards a better life, but at the end of the day it is your life and your decision to make. Your family will still love you in the end.

During graduate school, you may not see your family as often depending on distance but you will build a new family, your grad fam. A grad family is comprised of people that you meet during your graduate career. Your lab mates will become like siblings and your advisor like an academic parent (all on the assumption that you have joined a good lab). You will also meet other students in your department and from other departments through your classes. The students in my cohort, students that started around the same time, are some of my best friends. I have gone to their weddings and baby showers. They helped me grieve through losing a parent. Together we helped each other through our academic and personal struggles. There will also be other people that you work with on a regular basis like lab techs, admin, and building custodians that will become like family. Developing a grad fam was so crucial for my success. Going to the lab was like an episode of Cheers; Everyone knew my name and it became a place my home away from home. My recommendation is to diversify your grad fam. If you have only graduate students you risk creating a toxic family that focuses on only complaining about grad school, drinking too much, and work. You need other graduate students because they will understand your frustrations better than anyone else, but you will need to balance your family with individuals outside of academia. I highly recommend volunteering and finding local groups of interest to find these individuals. You will be thankful you did.

Diversity is important for your grad fam but unfortunately you may not find it in your department, program, or university. As the only Latina in most of my classes and in my department, while living in a city that lacks diversity, I really struggled with my identity. I missed being able to speak Spanish, to hear a Spanish radio station, and to find ingredients for family recipes. I would be lying if I said I never played Spanish music in my car out of fear of standing out. If you are from an underrepresented group, you may face some of these struggles.

The only way I got through it was by using it as my motivation; To be part of the 3% of Latinas that hold a PhD. Along with this example, I wrote all my motivators like my family, other Latinos, and my passion for science on a sticky note and kept it on my desk as a reminder. Another recommendation is to personalize your desk/lab bench. There will be times where you may not feel like you fit in for several different reasons, but your space will always be yours.

Feeling like you do not belong is called imposter syndrome. I would write a whole dissertation just on my experience with it but I will just suggest you look into it. This happens to most, if not all, graduate students, but more so in women and underrepresented groups. Just be aware that you will need to deal with this or else you may end up discounting yourself from opportunities that you can earn and deserve. My tips for dealing with imposter syndrome are 1) to acknowledge it and 2) be open to talking about it with other grad students because they will understand.

Grad school is the first time I ever heard a name for something I also experienced in undergrad, and grad school was also the first time for a whole plethora of other health issues. One thing I wish I had been told when starting grad school was life continues. Just because you are in graduate school does not mean that life stops happening. You will still deal with the everyday struggles of life such as family, relationships, society, and personal health. On top of these struggles you will also need to deal with stressors from grad school. Grad courses can be harder due to higher expectations, more freedom unlike undergrad courses, and trying to do research and teach while taking courses. Grad school will add stress to your already filled plate. Things I do not recommend doing to deal with stress are 1) abusing substances like alcohol (which is common in grad students), 2) withdraw into your work/students, 3) stop caring/become numb. These will all lead to mental health issues like anxiety, depression, and panic attacks.

During grad school I developed all of these. If you are already predisposed to mental health issues, grad school can be the fan that turns embers into flames. My first panic attack sent me to the ER because I thought I was having a heart attack. I thought panic attacks were people yelling and freaking out, not feeling like you cannot breathe and convulsing. Having so much stress on your plate forces you to find help to carry that plate. My helpers were counseling, my grad fam, and medication. Know that mental health issues are common and nothing to be ashamed of. Even post-defense depression is a thing! You are not too "weak" to handle grad school. You just have too much on your plate and need some help carrying it. My tips for dealing with mental health issues, whether new or if you had them before, is to not ignore it. Just because many grad students suffer from mental health issues does not mean you just suck it up. You cannot perform your best if your brain is not giving its best. Prepare yourself by finding a good support system, talking to your doctor about medications, and being completely open with your advisor. The relationship between you and your advisor can be beautiful but can also make your PhD ten times harder if you do not have a good one.

My final word of wisdom for current and incoming graduate students would be to know your worth. As graduate students, we do not need to be treated as work slaves or subjected to verbal or emotional abuse. If this happens, know you have options. You can switch labs. You can switch programs. You can switch schools. But never settle or think that "this is just how it is/has always been." You are worth so much more and owe it to yourself to stand up for yourself, your values, your needs, and your voice. You totally got this.

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APPENDIX A. SUPPLEMENTARY FIGURES (CHAPTER 2)

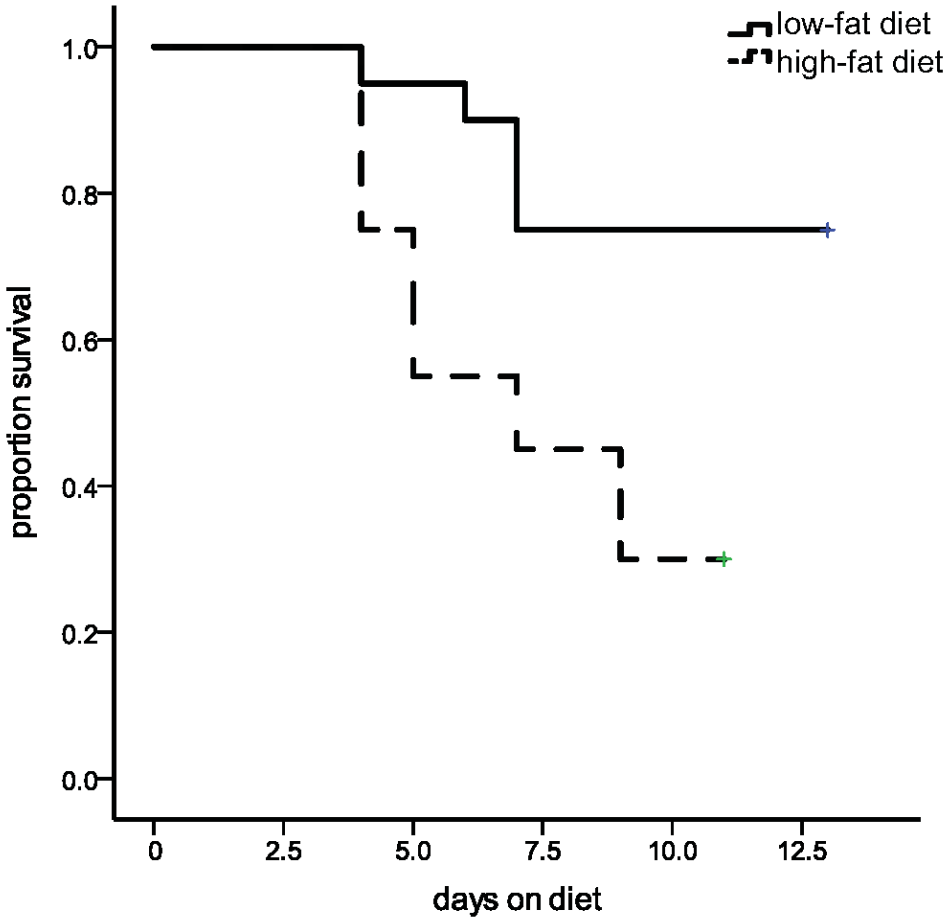

Figure A1. Survival of first instar caterpillars fed high-fat (dashed line) or low-fat (solid line) diets made with corn oil.

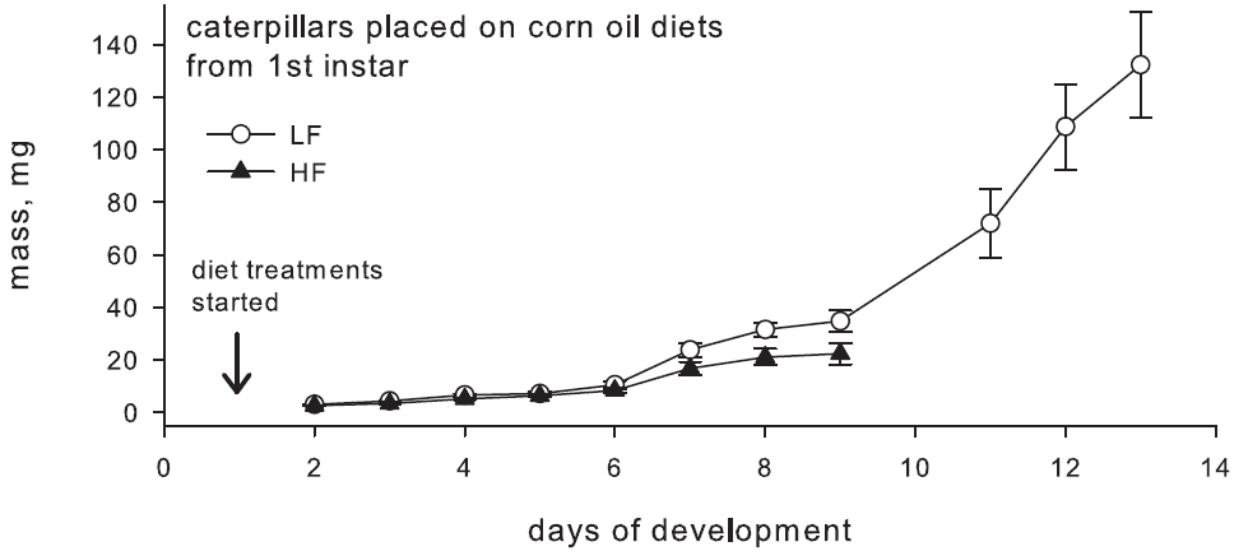

Figure A2. Body masses of caterpillars placed on HF (black triangles), MF (gray squares), or LF (open circles) diets in the first instar using corn oil.

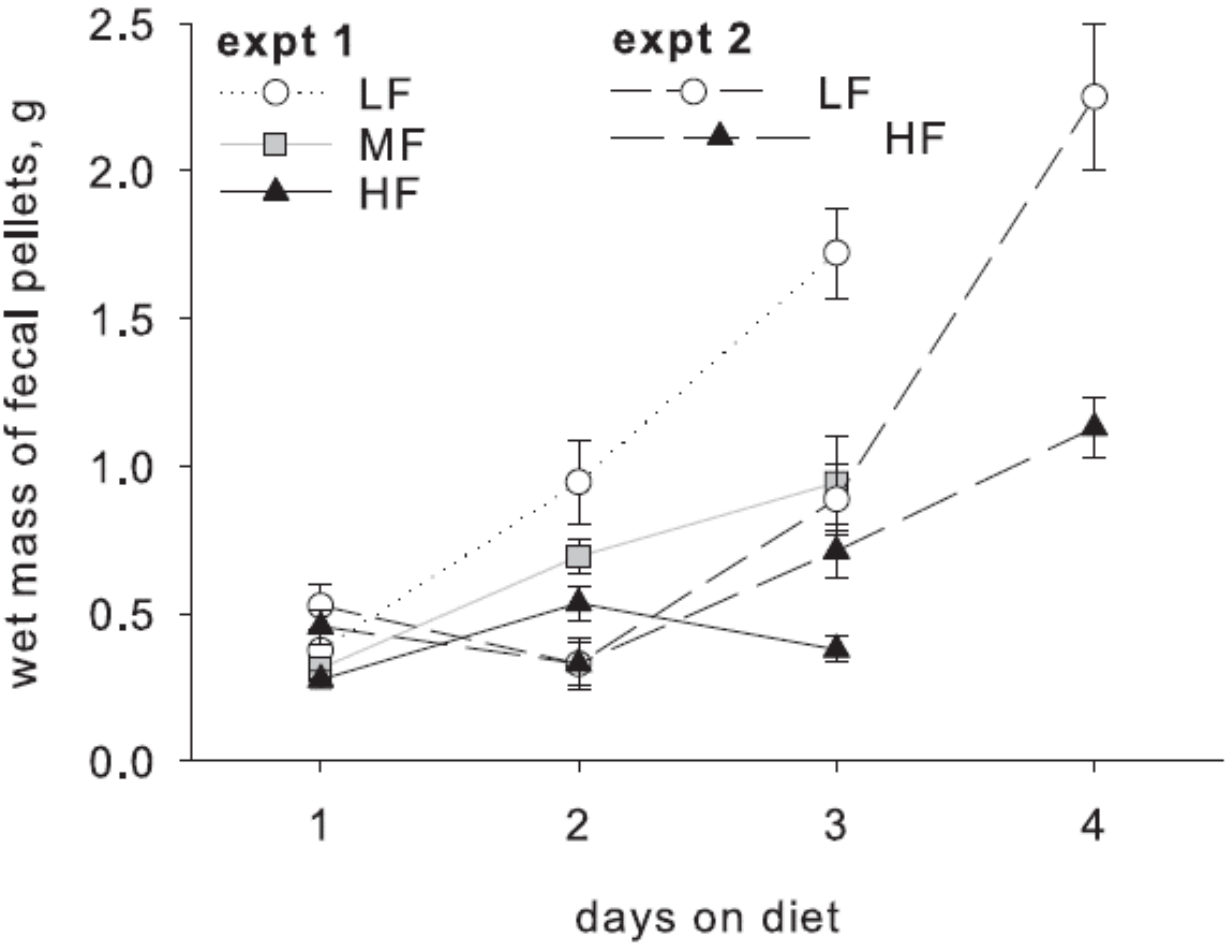

Figure A3. Wet masses of single fecal pellets of fifth instar *M. sexta* fed HF (black triangles), MF (gray squares), and LF (open circles) diets by experiment.

APPENDIX B. SUPPLEMENTARY FIGURES (CHAPTER 3)

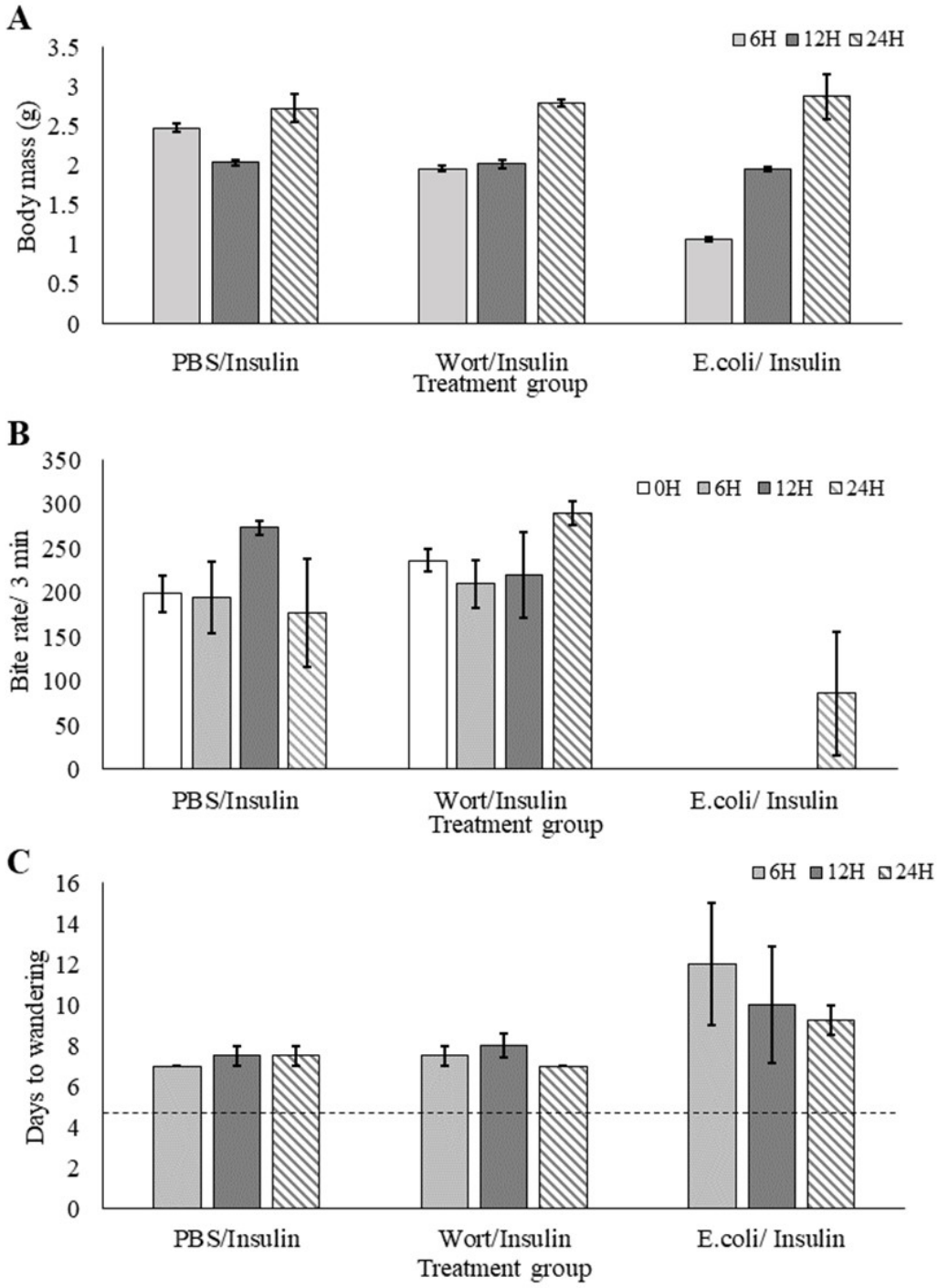

Figure B1. Effects of injection timing on insulin manipulation. Effects of the timing of injection on A) body mass, B) feeding, and C) developmental timing within treatment groups. Error bars indicate standard error. Dashed line indicates average days to wandering of 5 days.

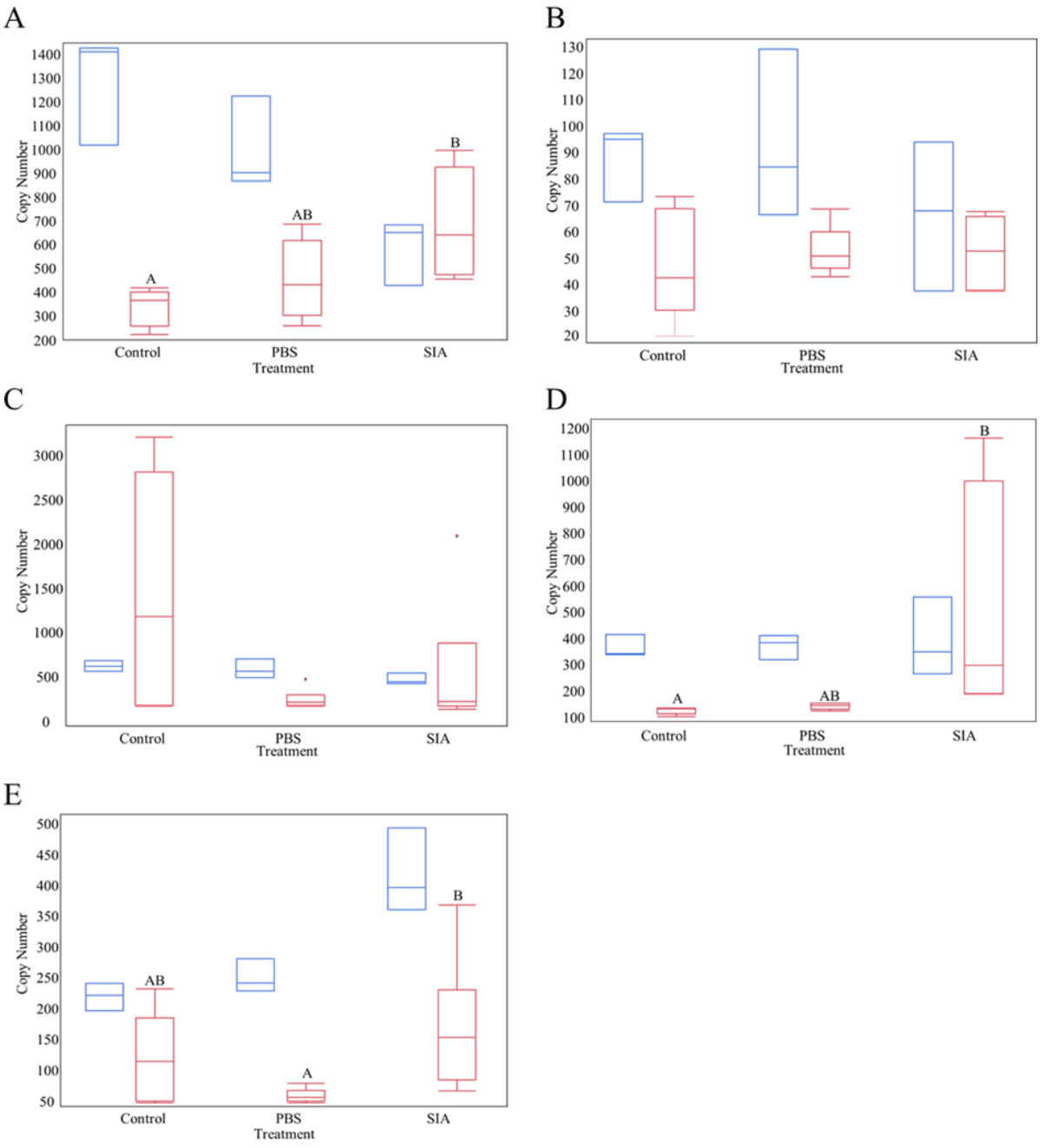

Figure B2. Gene expression for MAPK-JNK-p38 pathway targets. Expression of MAPK-JNK-p38 pathway targets A) *eiger*, B) *stress-activated protein kinase JNK*, C) *mitogen-activated protein kinase p38b-like*, D*) dSOR1*, and E) *mitogen-activated protein kinase 1* in fat body (red) and brain samples (blue). Different letters indicate significant differences between groups in fat body samples. There were no differences seen in brain samples.

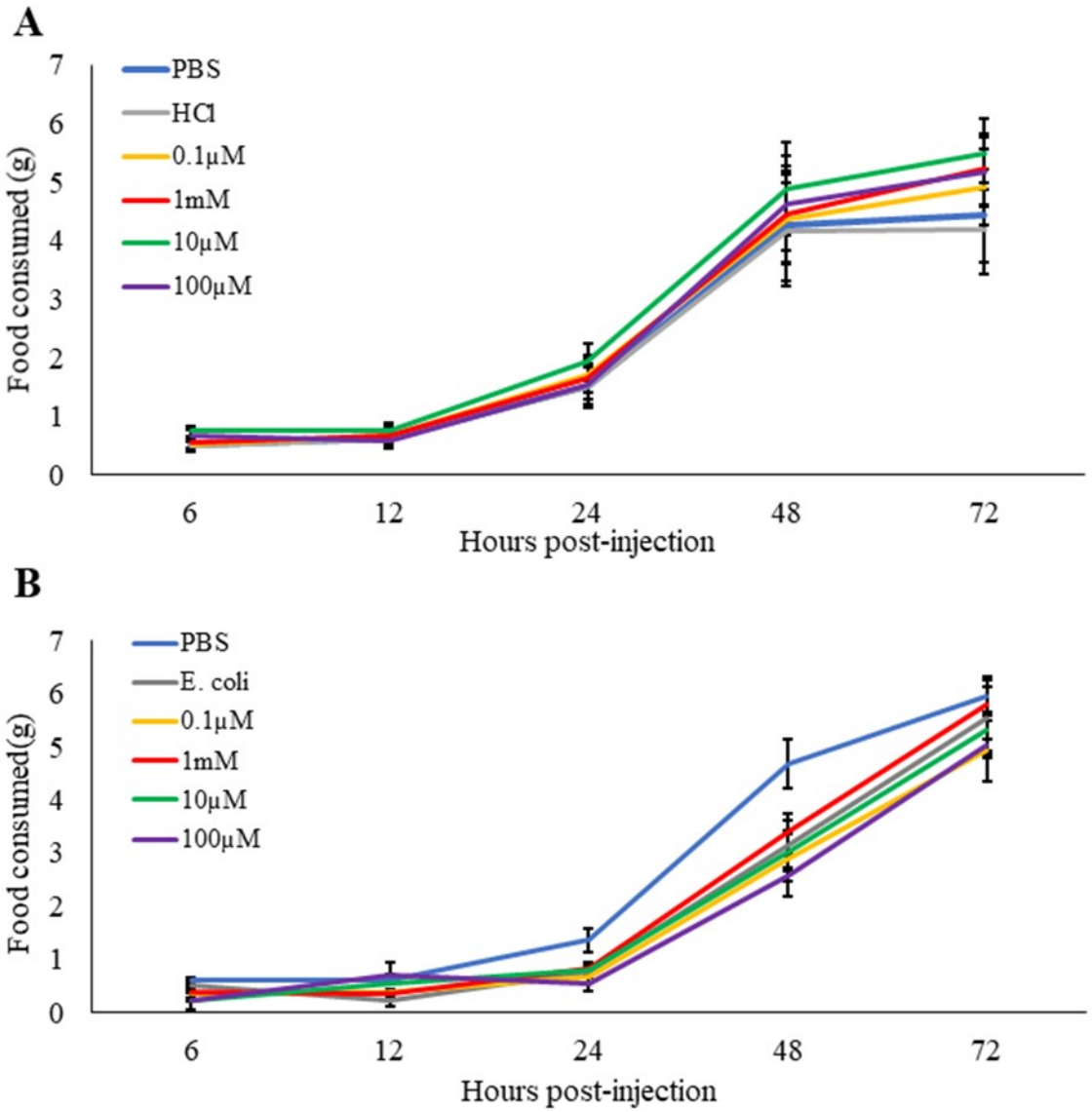

Figure B3. There were no dose effects of insulin and wortmannin on feeding in A) non-SIA and B) SIA larvae. Error bars indicate standard error.

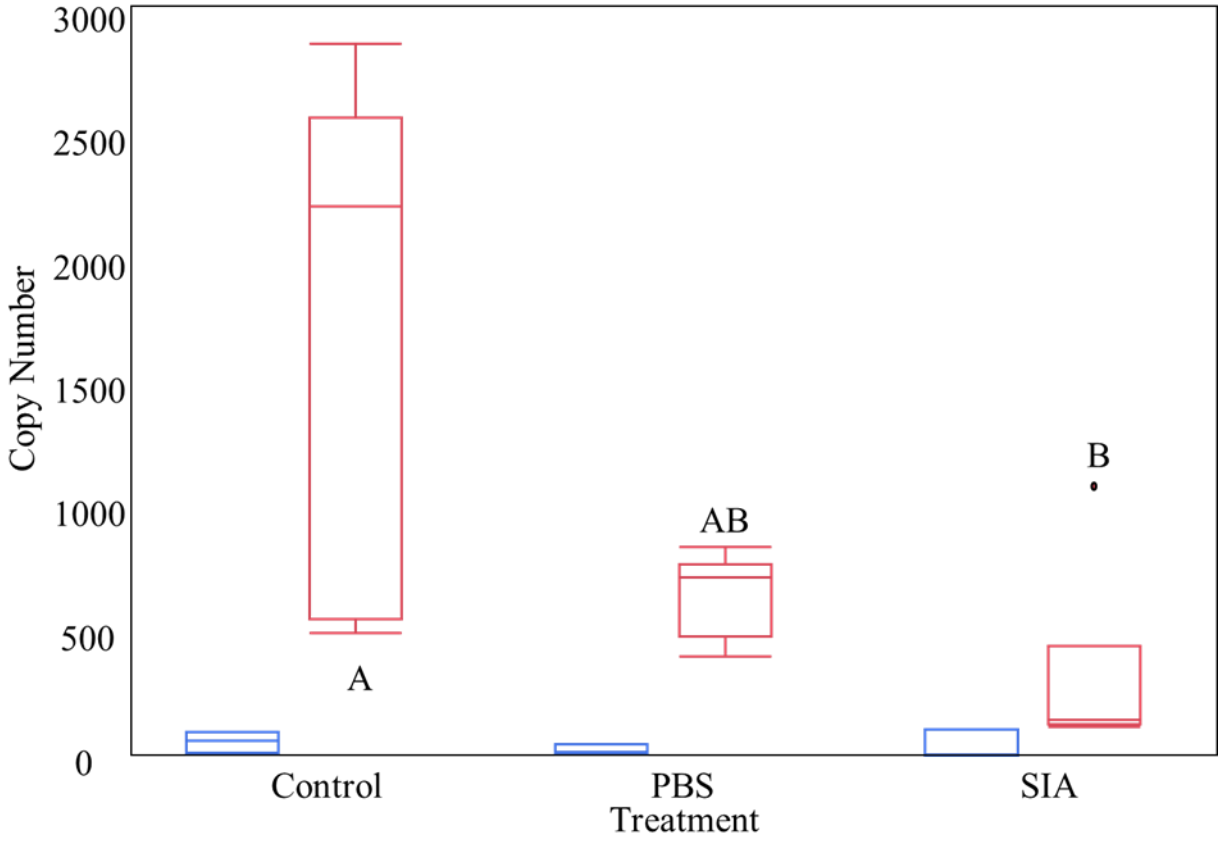

Figure B4. Gene expression for *gonadotropin-releasing hormone* (AKH) receptor. Expression of *gonadotropin-releasing hormone* (AKH) receptor in fat body (red) and brain samples (blue) during SIA. Different letters indicate significant differences between groups in fat body samples.

APPENDIX C. SUPPLEMENTARY TABLES (CHAPTER 3)

Table C1. Gene list used for NanoString custom probe design. Genes selected for nCounter analysis were derived from NCBI database for *M. sexta*. *indicates reference genes.

NCBI Accession #	Gene
NW_021942087.1	insulin receptor-like (LOC115443779)
NW_021942044.1	insulin receptor substrate 1 (LOC115441776)
NW_021942641.1	insulin-like growth factor 1 receptor (LOC115452173)
NW_021942008.1	insulin-related peptide 2-like (LOC115456431)
NW_021942048.1	insulin-related peptide 1-like (LOC115442025)
NW_021942104.1	ras-like protein 2 (LOC115444311)
NW_021942116.1	phosphatidylinositol 3-kinase catalytic subunit type 3 (LOC115444773)
NW_021942865.1	phosphatidylinositol 3-kinase regulatory subunit gamma (LOC115453059)
NW_021942105.1	serine/threonine-protein kinase mTOR-like (LOC115444378)
NW_021942105.1	serine/threonine-protein kinase mTOR (LOC115444377)
NW_021941982.1	glycogen synthase kinase-3 beta-like (LOC115442364)
NW_021941985.1	forkhead box protein O (LOC115446141)
NW_021941990.1	3-phosphoinositide-dependent protein kinase 1 (LOC115451287)
NW_021942044.1	phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase PTEN (LOC115441808)
NW_021941986.1	dual specificity mitogen-activated protein kinase kinase dSOR1 (ERK) (LOC115446913)
NW_021942119.1	GTP-binding protein Rheb homolog (LOC115444882)
NW_021942005.1	protein son of sevenless-like (LOC115456170)
NW_021941989.1	protein kinase C (LOC115449974)
NW_021942055.1	mitogen-activated protein kinase 1 (LOC115442325)
NW_021942205.1	gonadotropin-releasing hormone (AKH) receptor (LOC115447347)
NW_021942223.1	gonadotropin-releasing hormone receptor (LOC115447773)
NW_021942024.1	protein spaetzle (LOC115440684)
NW_021943174.1	protein toll-like (LOC115453732)
NW_021942515.1	myeloid differentiation primary response protein MyD88-like (LOC115451464)
NW_021942020.1	NF-kappa-B inhibitor cactus (LOC115440475)
NW_021942429.1	embryonic polarity protein dorsal-like (LOC115450820)
NW_021942039.1	protein eiger (LOC115441451)
NW_021942229.1	myc proto-oncogene protein (LOC115447895)
NW_021942222.1	tyrosine-protein kinase hopscotch (LOC115447758)
NW_021944682.1	protein pellino-like (LOC115454556)
NW_021942099.1	stress-activated protein kinase JNK (LOC115444202)

Table C1. Gene list used for NanoString custom probe design (continued). Genes selected for nCounter analysis were derived from NCBI database for *M. sexta*. *indicates reference genes.

NCBI Accession #	Gene
NW_021942273.1	mitogen-activated protein kinase p38b-like (LOC115448643)
NW_021942039.1	elongation factor 1-alpha (LOC115441475)*
NW_021954281.1	40S ribosomal protein S2 (LOC115455355)*
NW_021941996.1	40S ribosomal protein S3 (LOC115455570)*
NW_021942538.1	60S ribosomal protein L4 (LOC115451613)*
NW_021942416.1	60S ribosomal protein L8 (LOC115450656)*

Table C2. Summary statistics from Kruskal-Wallis test for the detection of significant differences in gene expression levels between treatment groups in brain samples. Summary statistics for each gene tested with nCounter in brain samples log transformed means± standard deviation for each treatment group, one-way test ChiSquare (χ^2) values, degrees of freedom (df), and p-values (p). * $p < 0.05$

Gene	Control	PBS	SIA	χ^2	df	p
3-phosphoinositide-dependent protein kinase 1	4.92±0.08	5.04±0.27	6.85±0.17	5.6000	2	0.0608
bombyxin-related peptide A-like	1.12±0.19	1.08±0.24	1.48±0.94	0.0889	2	0.9565
bombyxin-related peptide B-like	1.68±1.51	2.89±1.58	1.81±1.00	1.1556	2	0.5611
bombyxin-related peptide B-like 2	0.89±0.59	0.96±0.28	1.34±0.42	2.4889	2	0.2881
dSOR1 (ERK)	5.82±0.13	5.84±0.14	5.84±0.40	0.0000	2	1.0000
embryonic polarity protein dorsal-like	6.94±0.08	6.78±0.12	6.99±0.11	3.4667	2	0.1767
forkhead box protein O	4.63±0.15	4.73±0.07	4.99±0.15	5.9556	2	0.0509
glycogen synthase kinase-3 beta-like	4.40±0.05	4.24±0.04	4.05±0.28	5.6000	2	0.0608
gonadotropin-releasing hormone (AKH) receptor	3.64±1.18	2.69±1.13	2.54±1.93	1.0667	2	0.5866
gonadotropin-releasing hormone receptor	2.95±0.32	3.61±0.30	2.91±0.65	5.6000	2	0.0608
GTP-binding protein Rheb homolog	5.19±0.10	5.26±0.22	5.22±0.19	0.6222	2	0.7326
insulin receptor substrate 1	3.93±0.11	3.82±0.15	5.27±0.18	5.9556	2	0.0509
insulin receptor-like	3.99±0.04	3.85±0.40	4.84±0.66	4.3556	2	0.1133
insulin-like growth factor 1 receptor	3.17±0.27	3.87±0.21	3.24±0.29	5.6000	2	0.0608
insulin-related peptide 1-like	3.42±0.13	4.15±0.96	3.83±0.32	1.6889	2	0.4298
insulin-related peptide 2-like	3.55±0.43	4.29±0.65	4.16±0.45	3.2889	2	0.1931
M. sexta serine/threonine-protein kinase Mtor	5.46±0.13	5.4±0.05	5.3±0.43	0.0889	2	0.9565
mitogen-activated protein kinase 1	5.43±0.10	5.56±0.10	6.05±0.16	6.4889	2	0.039*
mitogen-activated protein kinase p38b-like	6.52±0.09	6.46±0.16	6.26±0.12	4.3556	2	0.1133
myc proto-oncogene protein	4.27±0.16	4.68±0.14	5.27±0.11	7.2000	2	0.0273*
myeloid differentiation primary response protein MyD88-like	5.50±0.04	5.19±0.19	5.35±0.30	3.2000	2	0.2019
NF-kappa-B inhibitor cactus	4.10±0.48	4.37±0.38	5.23±0.46	5.6000	2	0.0608
PTEN	4.64±0.08	4.79±0.08	4.11±0.32	7.2000	2	0.0273*
PI3K catalytic subunit type 3	3.86±0.34	3.77±0.12	3.93±0.38	0.2667	2	0.8752
PI3K regulatory subunit gamma	5.52±0.24	5.41±0.25	5.28±0.20	2.2222	2	0.3292
protein eiger	7.15±0.19	6.90±0.19	6.36±0.25	6.4889	2	0.0390*
protein kinase C	5.74±0.09	5.76±0.16	5.65±0.27	0.3556	2	0.8371
protein pellino-like	6.05±0.12	5.84±0.10	5.93±0.08	3.8222	2	0.1479
protein son of sevenless-like	5.10±0.01	5.01±0.23	4.85±0.44	0.8000	2	0.6703
protein spaetzle	4.78±0.14	4.89±0.23	4.85±0.07	1.0667	2	0.5866
protein toll-like	1.96±0.11	2.39±0.51	4.41±0.99	5.9556	2	0.0509
ras-like protein 2	6.56±0.08	6.51±0.15	6.46±0.08	2.2222	2	0.3292
serine/threonine-protein kinase mTOR-like	3.60±0.25	4.09±0.38	3.37±1.04	2.4889	2	0.2881
stress-activated protein kinase JNK	4.43±0.18	4.46±0.35	4.08±0.49	1.8667	2	0.3932
tyrosine-protein kinase hopscotch	4.72±0.09	4.59±0.09	4.70±0.12	2.4889	2	0.2881

Table C3. Summary statistics from Kruskal-Wallis test for the detection of significant differences in gene expression levels between treatment groups in fat body samples. Summary statistics for each gene tested with nCounter in fat body samples log transformed means± standard deviation for each treatment group, one-way test ChiSquare (χ^2) values, degrees of freedom (df), and p-values (p). * $p < 0.05$* $p < 0.05$, ** $p < 0.01$

Gene	Control	PBS	SIA	χ^2	df	p
3-phosphoinositide-dependent protein kinase 1	4.62±0.37	4.14±0.24	6.91±0.28	12.74	2	0.001**
bombyxin-related peptide A-like	1.23±0.75	0.41±0.28	0.78±0.37	7.01	2	0.030*
bombyxin-related peptide B-like	0.96± 0.88	0.38±0.45	0.57±0.46	1.47	2	0.479
bombyxin-related peptide B-like 2	1.27±0.33	0.36±0.36	0.9±0.37	8.32	2	0.016*
dSOR1 (ERK)	4.58±0.14	4.75±0.11	5.86±0.87	12.44	2	0.002**
embryonic polarity protein dorsal-like	6.42±0.37	5.94±0.29	6.61±0.52	7.04	2	0.030*
forkhead box protein O	4.85±0.38	4.39±0.29	5.0±0.18	7.16	2	0.028*
glycogen synthase kinase-3 beta-like	3.33±0.91	2.28±0.41	2.91±0.72	6.77	2	0.034*
gonadotropin-releasing hormone (AKH) receptor	7.20±0.83	6.46±0.28	5.34±0.84	7.15	2	0.028*
gonadotropin-releasing hormone receptor	3.62±1.59	2.29±0.46	2.19±0.55	2.50	2	0.286
GTP-binding protein Rheb homolog	5.52±1.26	4.15±0.43	4.79±0.79	5.64	2	0.060
insulin receptor substrate 1	4.40±0.84	3.36±0.31	5.53±0.60	10.72	2	0.005**
insulin receptor-like	3.53±0.38	3.00±0.28	4.61±0.22	13.07	2	0.002**
insulin-like growth factor 1 receptor	0.85±0.70	0.22±0.29	0.38±0.50	3.81	2	0.149
insulin-related peptide 1-like	1.19±0.52	1.07±0.62	0.92±0.16	1.85	2	0.396
insulin-related peptide 2-like	0.82±0.74	0.27±0.25	0.48±0.44	1.76	2	0.415
M. sexta serine/threonine-protein kinase Mtor	4.19±0.55	4.28±0.27	4.80±0.40	6.32	2	0.043*
mitogen-activated protein kinase 1	4.69±0.62	4.20±0.17	5.05±0.56	6.63	2	0.036*
mitogen-activated protein kinase p38b-like	6.79±1.3	5.67±0.30	5.99±0.89	1.29	2	0.524
myc proto-oncogene protein	4.96±0.93	4.9±0.37	4.55±0.71	1.83	2	0.401
MyD88-like	4.80±0.36	4.61±0.27	5.71±0.65	11.70	2	0.003**
NF-kappa-B inhibitor cactus	3.50±0.77	2.70±0.25	5.35±0.73	12.44	2	0.002**
PTEN	5.07±1.13	4.02±0.36	3.47±0.72	7.94	2	0.019*
PI3K catalytic subunit type 3	3.87±0.52	3.34±0.31	3.65±0.43	3.48	2	0.175
PI3K regulatory subunit gamma	5.61±1.12	4.60±0.48	4.76±0.99	2.53	2	0.282
protein eiger	5.8±0.25	6.07±0.36	6.49±0.33	9.02	2	0.011*
protein kinase C	5.11±1.04	3.99±0.38	4.67±0.77	4.16	2	0.125
protein pellino-like	5.23±0.81	4.33±0.26	6.21±0.83	9.90	2	0.007**
protein son of sevenless-like	4.14±0.36	4.28±0.24	4.36±0.21	0.94	2	0.625
protein spaetzle	3.96±0.57	3.63±0.27	4.37±0.58	6.72	2	0.035*
protein toll-like	1.46±0.57	1.98±0.14	3.27±0.83	10.85	2	0.004**
ras-like protein 2	6.51±1.08	5.43±0.28	5.83±0.80	1.90	2	0.387
serine/threonine-protein kinase mTOR-like	2.87±0.38	3.00±0.06	2.99±0.23	1.35	2	0.510
stress-activated protein kinase JNK	3.71±0.54	3.90±0.18	3.87±0.28	0.44	2	0.803
tyrosine-protein kinase hopscotch	5.28±0.65	4.65±0.33	5.77±0.75	7.09	2	0.028*

APPENDIX D. SUPPLEMENTARY FIGURES (CHAPTER 4)

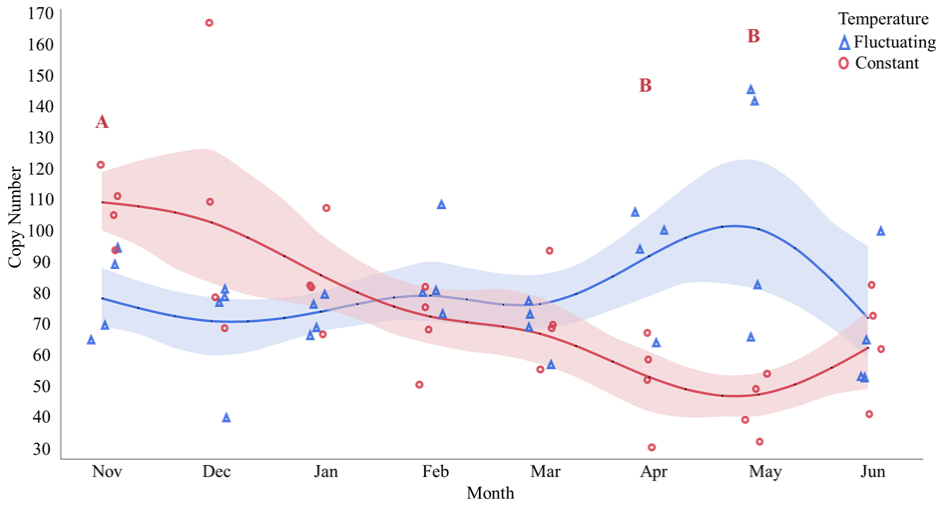

Figure D1. Gene expression for AKT-interacting protein-like. There was no significant difference in gene expression for AKT-interacting protein-like between temperature treatments. However, within the constant temperature group, expression during April and May were significantly different from November. Months with different letters are significantly different. Letter colors correspond to temperature treatment. 95% confidence intervals are shown in shaded areas.

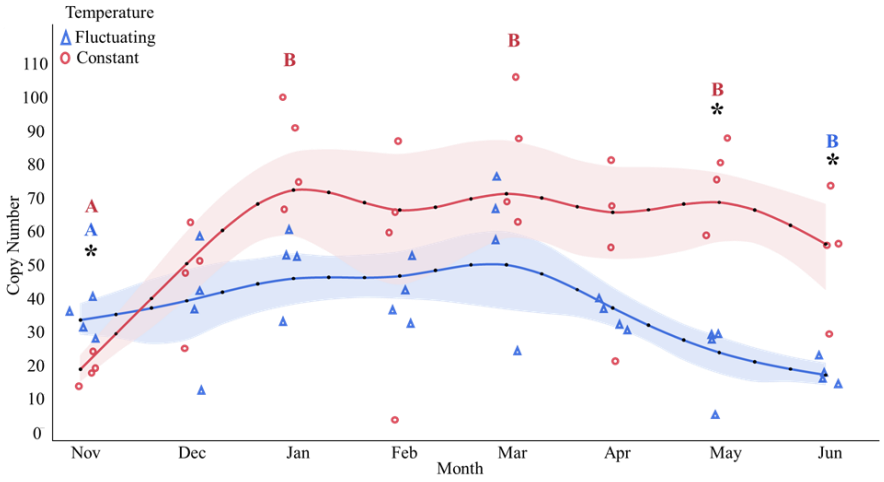

Figure D2. Gene expression for cyclin D. Gene expression for cyclin D was significantly different between temperature treatments in November, May, and June. Within treatments, fluctuating temperature showed a similar trend with November being significantly different from June, whereas samples kept in a constant temperature showed November being significantly different from January, March, and May. Months with different letters are significantly different. Letter colors correspond to temperature treatment. Asterisks indicate significant differences between temperature treatments at a given month. 95% confidence intervals are shown in shaded areas.

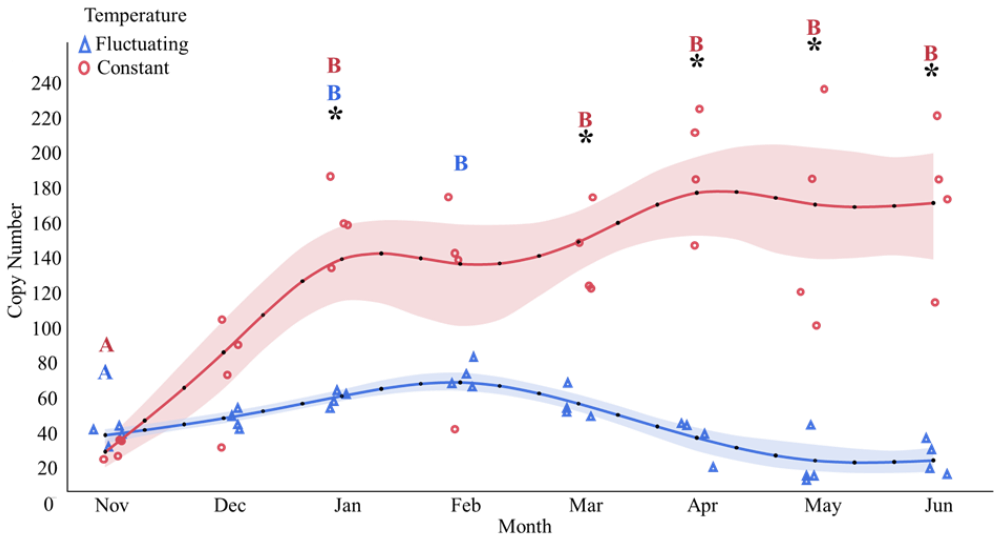

Figure D3. Gene expression for cyclin E. Gene expression for cyclin E was significantly different between temperature treatments from March to June. Within treatments, fluctuating temperature showed November being significantly different from January and February, whereas samples kept in a constant temperature showed November being significantly different from January, and March through June. Months with different letters are significantly different. Letter colors correspond to temperature treatment. Asterisks indicate significant differences between temperature treatments at a given month. Shaded areas indicate 95% confidence intervals.

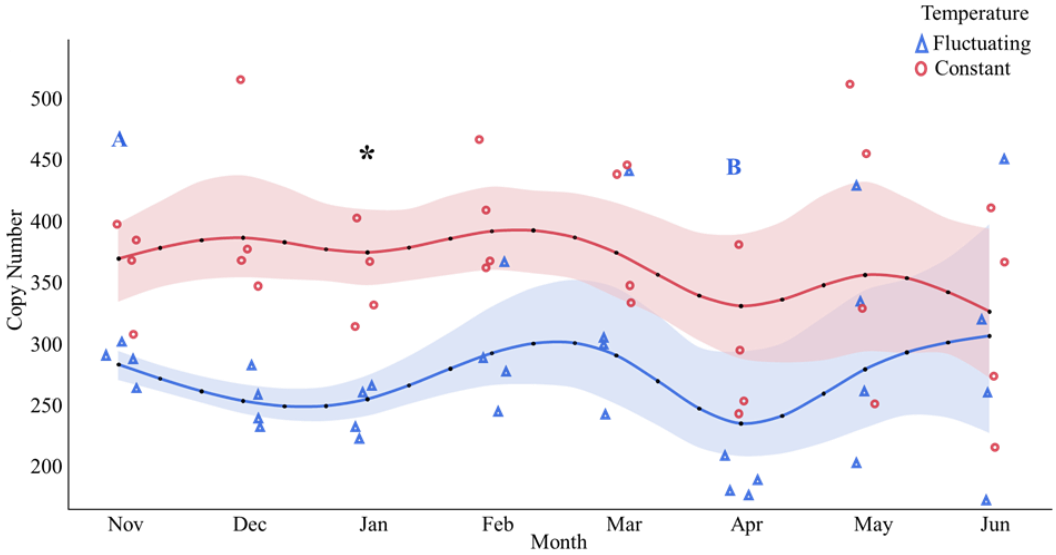

Figure D4. Gene expression for cyclin G. Gene expression for cyclin G was on significantly different between temperature treatments during the month of January. The month of April was only significantly different from November for samples kept in fluctuating temperatures. Months with different letters are significantly different. Letter colors correspond to temperature treatments. Asterisks indicate significant differences between temperature treatments at a given month. 95% confidence intervals are shown in shaded areas.

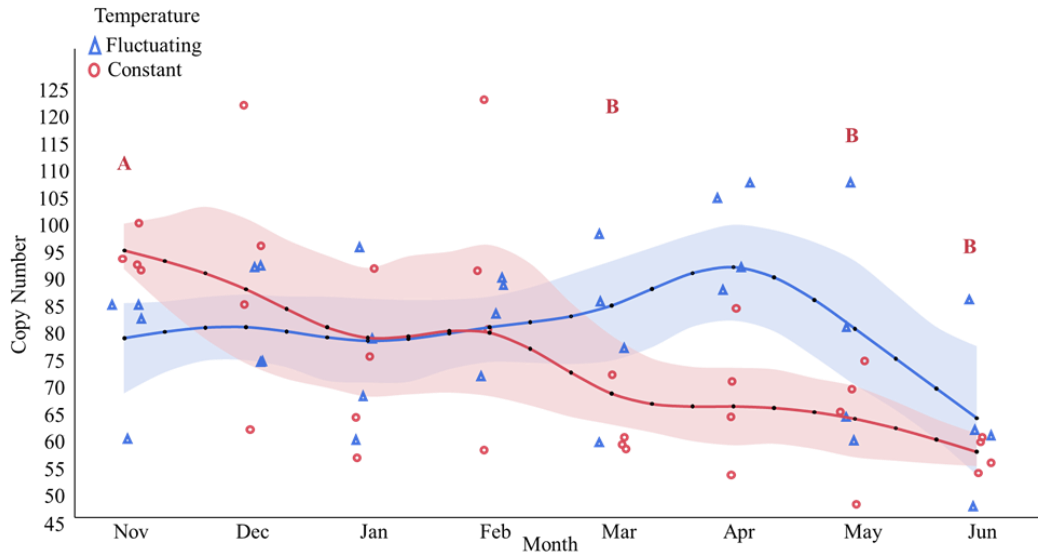

Figure D5. Gene expression for ERK. Gene expression for ERK was only significantly different in the constant temperature group. In this group, November was significantly different from March, May, and June. Months with different letters are significantly different. Letter colors correspond to temperature treatment. 95% confidence intervals are shown in shaded areas.

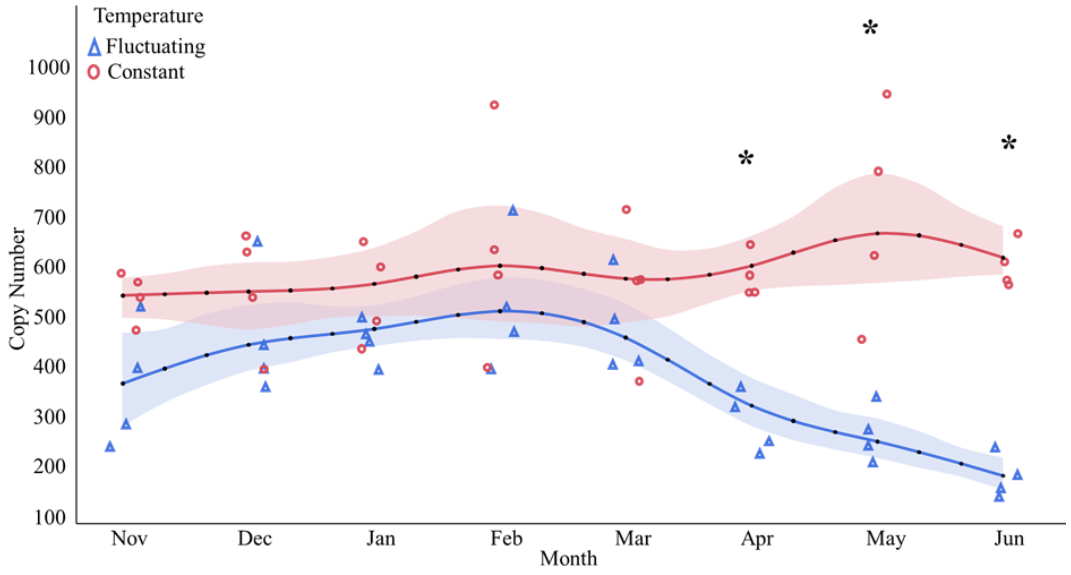

Figure D6. Gene expression for FOXO. FOXO gene expression was significantly different between temperature treatments during the months of April through June. Asterisks indicate significant differences between temperature treatments at a given month. 95% confidence intervals are shown in shaded areas.

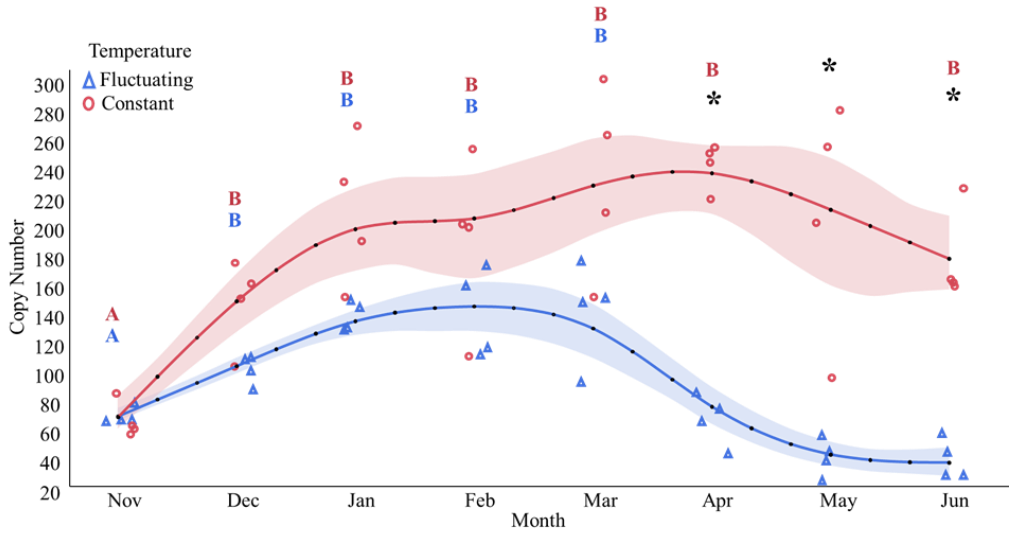

Figure D7. Gene expression for GAB2. GAB2 gene expression was significantly different between temperature treatments from April through June. Within each treatment, February through March were significantly different from November in fluctuating temperatures, and all but May were significantly different in samples kept at a constant temperature. Months with different letters are significantly different. Letter colors correspond to temperature treatments. Asterisks indicate significant differences between temperature treatments at a given month. 95% confidence intervals are shown in shaded areas.

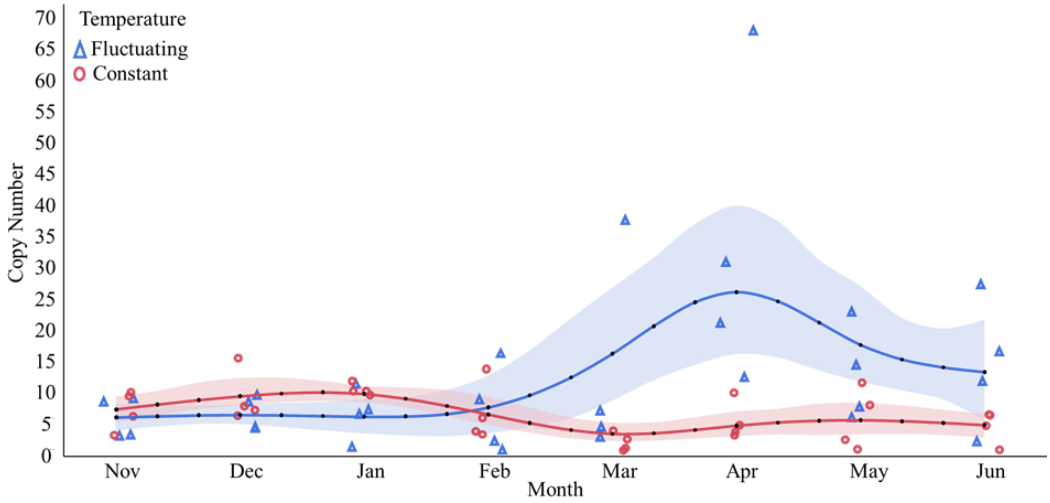

Figure D8. Gene expression for IGF. Gene expression for IGF was not significantly different by temperature treatment or across months relative to November. 95% confidence intervals are shown in shaded areas.

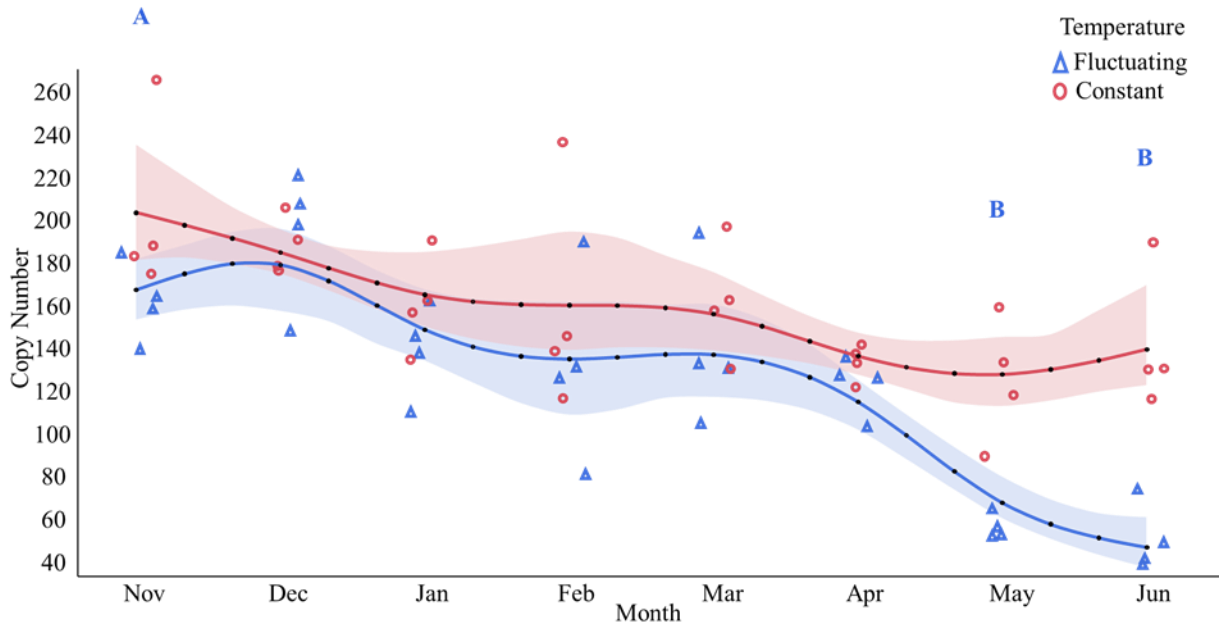

Figure D9. Gene expression for insulin-like receptor. During the months of May and June, gene expression for insulin-like receptor was significantly different between temperature treatments, and significantly different from November in fluctuating temperatures. Months with different letters are significantly different. Letter colors correspond to temperature treatments. Asterisks indicate significant differences between temperature treatments at a given month. 95% confidence intervals are shown in shaded areas.

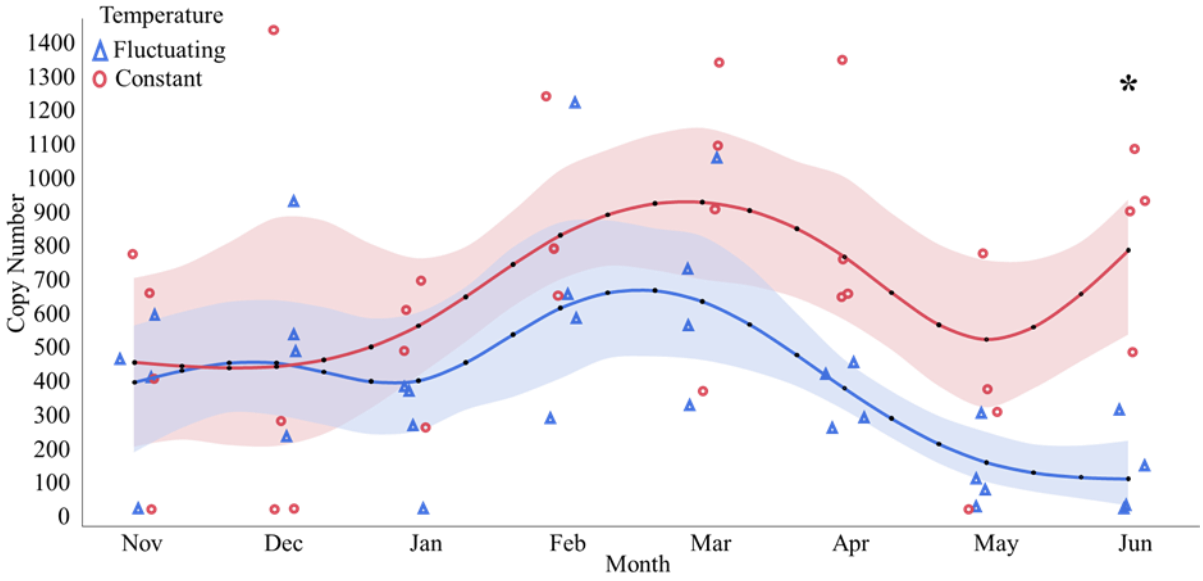

Figure D10. Gene expression for insulin-receptor like. Gene expression for insulin-receptor like was only significantly different in June between temperature treatments, as indicated by an asterisk. 95% confidence intervals are shown in shaded areas.

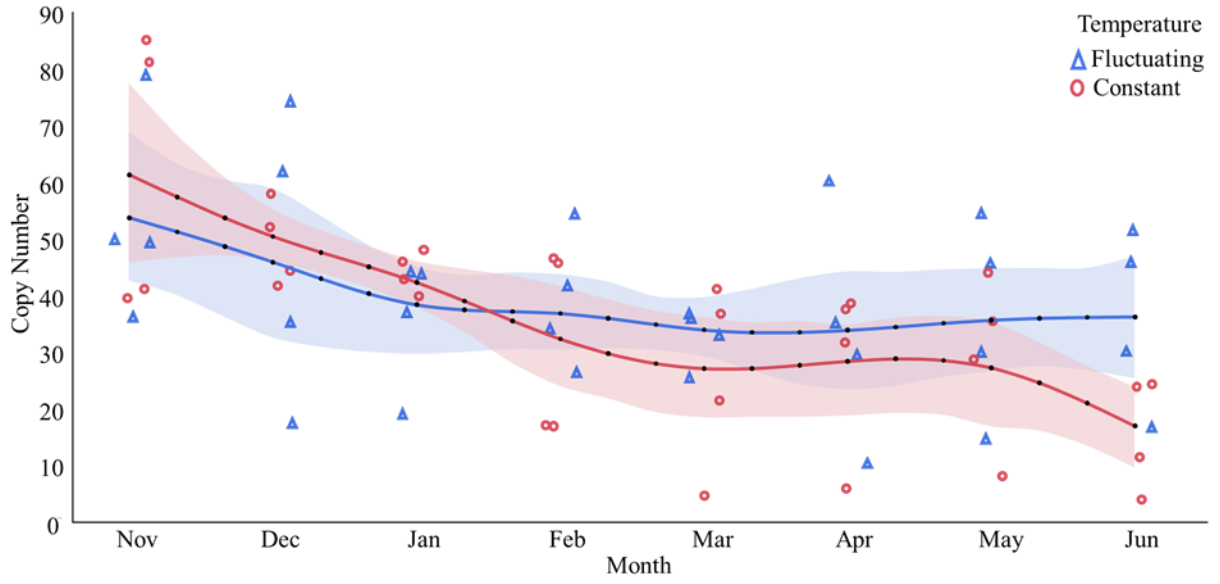

Figure D11. Gene expression for MAPK1. Gene expression for MAPK1 was not significantly different by temperature treatment or across months relative to November. 95% confidence intervals are shown in shaded areas.

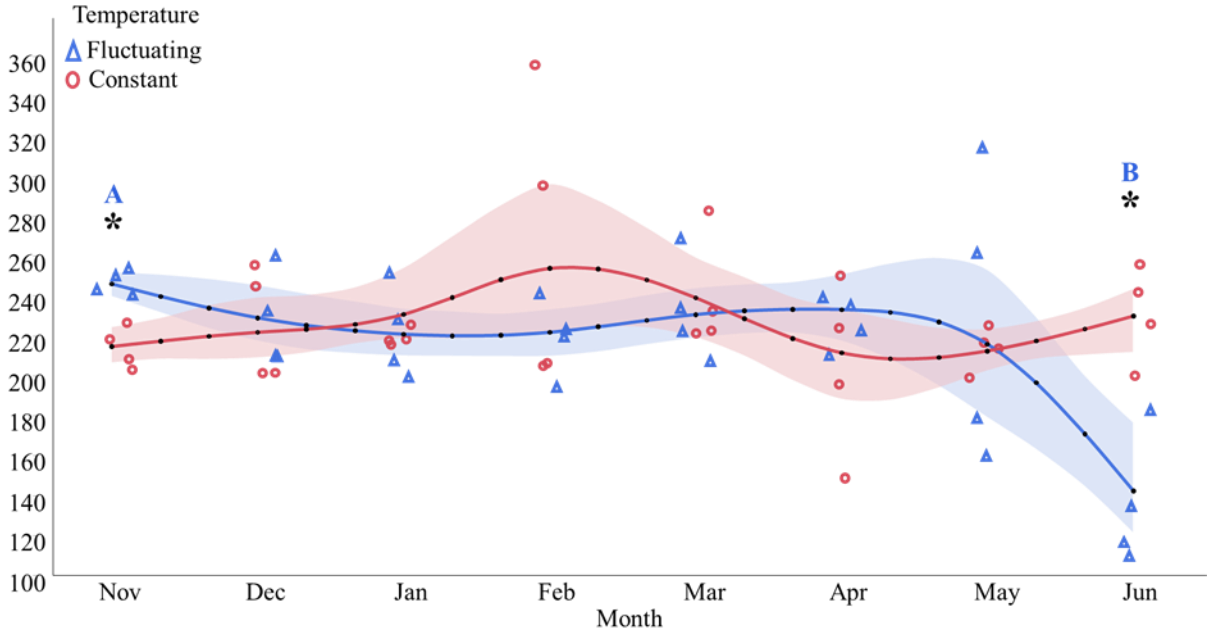

Figure D12. Gene expression for MAPK14B. Significant differences in gene expression for MAPK14B between temperature treatments was seen in November and June. Within the fluctuating temperature group, June was also significantly different than November. Months with different letters are significantly different. Letter colors correspond to temperature treatments. Asterisks indicate significant differences between temperature treatments at a given month. 95% confidence intervals are shown in shaded areas.

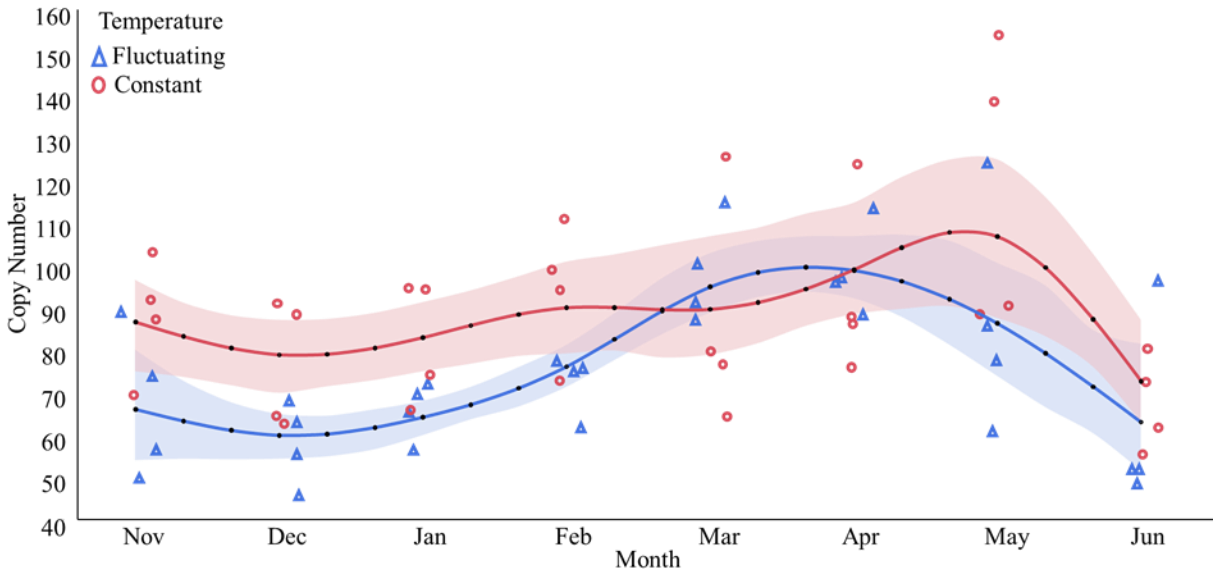

Figure D13. Gene expression for PI3K catabolic subunit. Gene expression for PI3K catalytic subunit was not significantly different by temperature treatment or across months relative to November. 95% confidence intervals are shown in shaded areas.

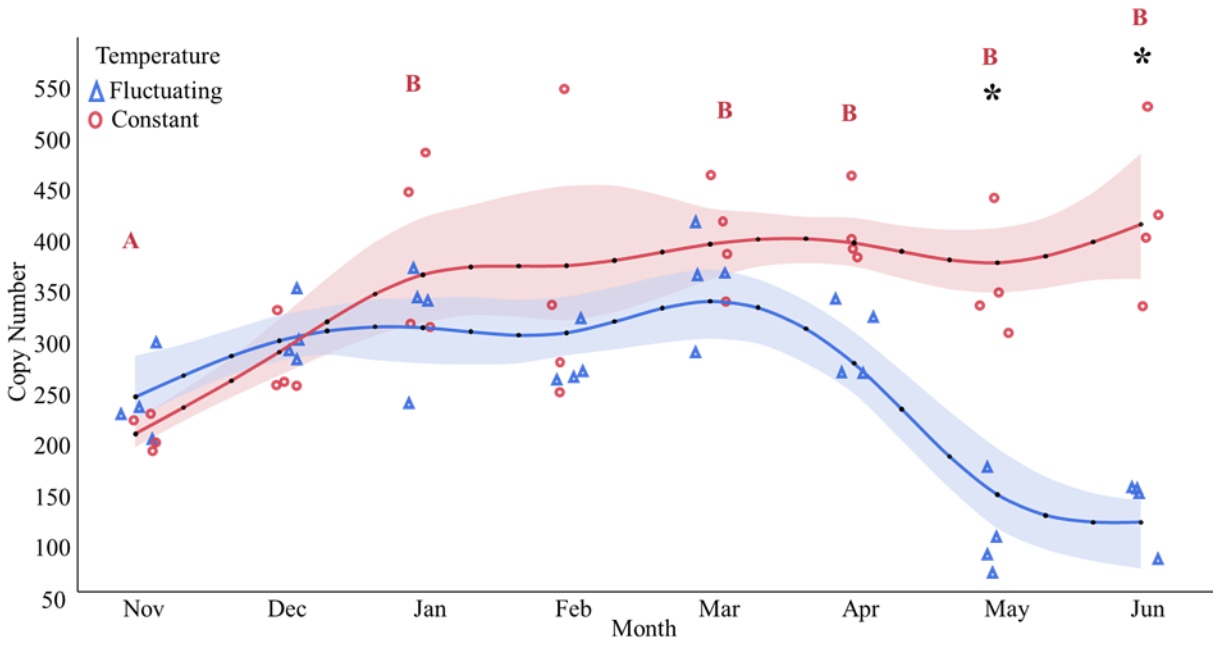

Figure D14. Gene expression for PI3K regulatory subunit. During May and June, gene expression for PI3K regulatory subunit was significantly different between temperature treatments. Within the constant temperature group, November was significantly different than January and March through June. Months with different letters are significantly different. Letter colors correspond to temperature treatments. Asterisks indicate significant differences between temperature treatments at a given month. 95% confidence intervals are shown in shaded areas.

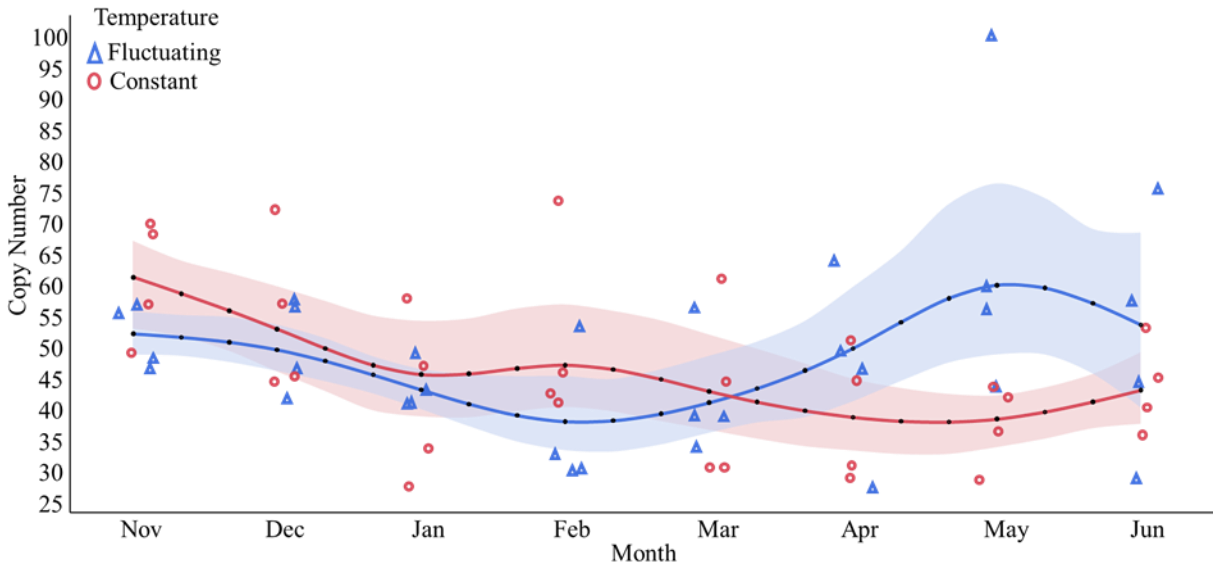

Figure D15. Gene expression for PTEN. Gene expression for PTEN was not significantly different by temperature treatment or across months relative to November. 95% confidence intervals are shown in shaded areas.

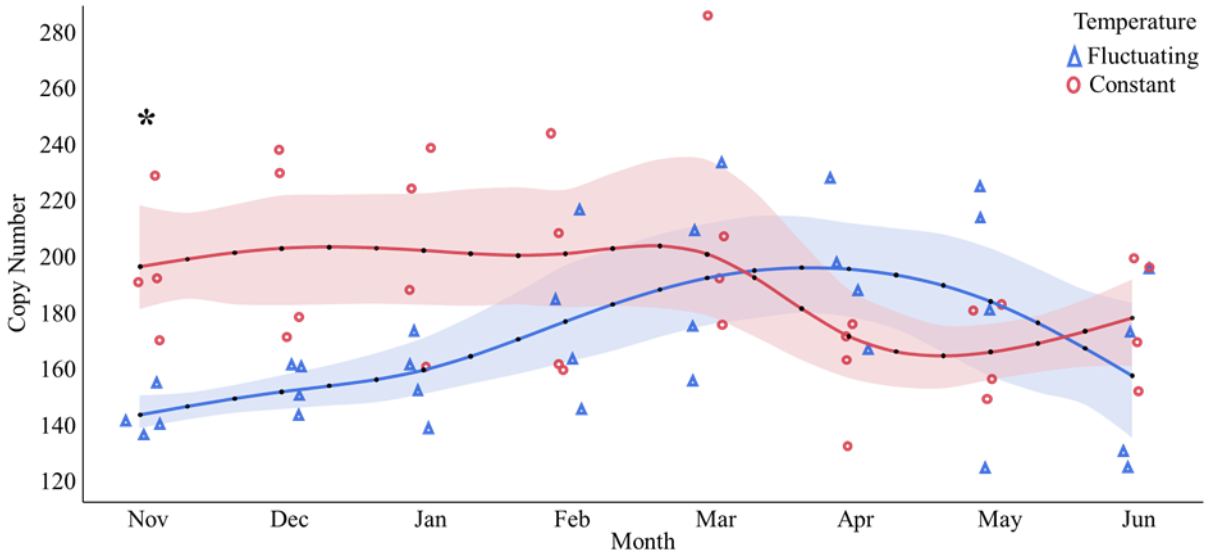

Figure D16. Gene expression for RAS2. During the month of November, gene expression for RAS2 was significantly different between temperature treatments, as indicated by an asterisk. 95% confidence intervals are shown in shaded areas.

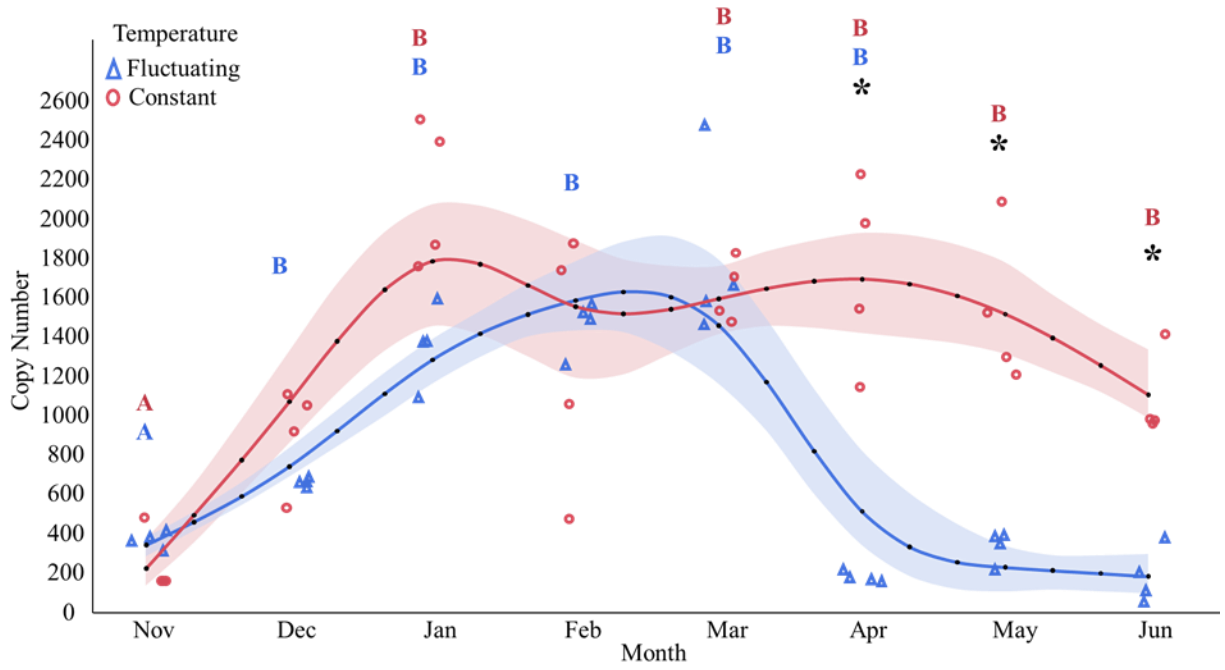

Figure D17. Gene expression for samui. Samui gene expression was significantly different between temperature treatments during the months of April through June. Within each temperature treatments, November was significantly different from January through April in the fluctuating temperatures and from January and March through June in the constant temperatures. Months with different letters are significantly different. Letter colors correspond to temperature treatments. Asterisks indicate significant differences between temperature treatments at a given month. 95% confidence intervals are shown in shaded areas.

APPENDIX E. SUPPLEMENTARY TABLES (CHAPTER 4)

Table E1. List of target genes for qPCR. Genes selected for qPCR were derived from NCBI database for *M. rotundata.*

Gene	NCBI GeneID
Insulin-like growth factor I (IGF)	100880980
Insulin receptor-like (InR)	100879880
Protein L-Myc-1b (MYC)	100879259
Serine/threonine-protein kinase (mTOR)	100880773
Glycogen synthase kinase-3 beta-like (GSK3-β)	100882062
Forkhead box protein O (FOXO)	100882630
Phosphatidylinositol 3-kinase regulatory subunit alpha (PI3K)	100878497
Pyruvate dehydrogenase kinase 1 (PDK-1)	100876315
Mitogen-Activated Protein Kinase 1 (MAPK1)	100878492
Mitogen-Activated Protein Kinase 14B-like (MAPK14B)	100881316

Table E2. Primer sequences for qPCR. Sequences for forward and reverse primers were designed using Primer Quest Tool from Integrated DNA Technologies. Top sequences are forward primers and bottom sequences are reverse primers. Certain genes had several conserved regions, therefore two primers were designed. XLOC stands for transcript number from Yocum *et. al* 2018 used for reference genes.

Gene	Primer sequence
Insulin receptor-like (InR)	5'-GAAGGTTCGAGCTACCAGTTT-3'
	5'-GCCAGAAGATCAGCCAGAAT-3'
Insulin-like growth factor I (IGF)	5'-CTCCGTGATCATAAGCCTTCC-3'
	5'-GGGACTTGGTCCAGGTTATTC-3'
Protein L-Myc-1b (MYC)	5'-GACCATTGCTACCACCTCAATC-3'
	5'-AGGTCACTACGTCGATCTCTTC-3'
Serine/threonine-protein kinase (mTOR)	5'-ACATACACACGGTGACGATTTAT-3'
	5'-GACATAACAGCGAGAGAACGAG-3'
	5'- AGAGCATAGCGACACATTGG-3'
	5'- GCGCTTCTGGCTGATTAGTAT-3'
Glycogen synthase kinase-3 beta-like (GSK3-β)	5'-GGAAGAACTTGTCGCCATCA-3'
	5'- CAGTTTCACGATGTTGCAGTG-3'
	5'-CGAGATCTTCCTCCGTTGTTT-3'
	5'-TCTGCATGTACTTCGGCTTTAG-3'

Table E2. Primer sequences for qPCR. Sequences for forward and reverse primers were designed using Primer Quest Tool from Integrated DNA Technologies (continued). Top sequences are forward primers and bottom sequences are reverse primers. Certain genes had several conserved regions, therefore two primers were designed. XLOC stands for transcript number from Yocum et. al 2018 used for reference genes.

Gene	Primer sequence
Forkhead box protein O (FOXO)	5'-ACACCGACCTACACACCTTCTT -3'
	5'-GGTGGACCTGTTTGTTGCTGAT -3'
Phosphatidylinositol 3-kinase regulatory subunit alpha (PI3K)	5'-GGCTCGTTGCTCATCCTAAA-3'
	5'-GTCGCCAGGGCACTTAAA-3'
Pyruvate dehydrogenase kinase 1 (PDK-1)	5'-GCGTGTACAGCCGGGAATTTA-3'
	5'-CCACCGCTTCTCCTTTCTTCTTT-3'
Mitogen-Activated Protein Kinase 1 (MAPK1)	5'-TGCTTACGGGATGGTCGTATCT-3'
	5'-GTTCTCTGGCTATAAGTCTGATGT TCAAA-3'
Mitogen-Activated Protein Kinase 14B (MAPK14B)	5'-ACACACGATGGATGTAGGGTTT-3'
	5'-CGTATGAGACAGTACTTTACAGACACTT-3'
	5'-CAGACGAACCGGTATCTCTACCATA -3'
	5'-CGGTGAAGACATTGCAGGTAGTT-3'
Reference gene #1: XLOC_004935	5'-GGATGATTGTACGGTCGAAACT-3'
	5'-CAACCGCATCCTTACTCTATCG-3'
Reference gene #2: XLOC_005046	5'-TACGACTGGTTTCAGGACATTT-3'
	5'-CGTGTTTCGGTTCGCTTATTG-3'
Reference gene #3: XLOC_005958	5'-TCCGAACATTGTACCACGAAG-3'
	5'-TCCGCGAGTAGCTACGATAA-3'

Table E3. List of Target Genes for NanoString. Genes selected for NanoString were derived from NCBI database for *M. rotundata.* Genes in blue were selected based on Yocum *et. al* 2018 as stably expressed through overwintering, and used as reference genes.

Gene	NCBI GeneID
Insulin Receptor-Like	100879880
Insulin Receptor Substrate 1	100879231
Insulin-Like Growth Factor I	100880980
Insulin-Like Receptor	100882691
Ras-Like Protein 1	100876644
Ras-Like Protein 2	100879495
Phosphatidylinositol 3-Kinase Regulatory Subunit Alpha (P85)	100878497
Phosphatidylinositol 3-Kinase Catalytic Subunit Type 3	100882290
Protein L-Myc-1b	100879259
Serine/Threonine-Protein Kinase (mTOR)	100880773
Glycogen Synthase Kinase-3 Beta-Like	100882062
Forkhead Box Protein O	100882630
AKT-Interacting Protein-Like	100883676
3-Phosphoinositide-Dependent Protein Kinase 1 (PDK1)	100876379
Phosphatidylinositol 3,4,5-Trisphosphate 3-Phosphatase And Dual-Specificity Protein Phosphatase (PTEN)	100883081
GRB2-Associated-Binding Protein 2	100883689
Tether Containing UBX Domain For GLUT4	100882950
Dual Specificity Mitogen-Activated Protein Kinase Kinase Dsor1 (ERK)	105664328
GTP-Binding Protein Rheb Homolog (RHEB)	100880016
Protein Son Of Sevenless (SOS)	100883790
Protein Kinase C	100879571
Mitogen-Activated Protein Kinase 1 (MAPK1)	100878492
Mitogen-Activated Protein Kinase 14B-Like (MAPK14B)	100881316
Adipokinetic Prohormone Type 2-Like	105663337
Gonadotropin-Releasing Hormone II Receptor (AKH)	100882851
Samui	100881147
Cyclin-K	100875496
Cyclin-D	100876021
Cyclin-E	100880332
Cyclin-G	100884096

Table E3. List of Target Genes for NanoString. Genes selected for NanoString were derived from NCBI database for *M. rotundata* (continued). Genes in blue were selected based on Yocum *et. al* 2018 as stably expressed through overwintering, and used as reference genes.

Gene	NCBI GeneID
Megachile rotundata Actin-5C	100882031
Megachile rotundata Tubulin Alpha-1 Chain-Like	100880305
Megachile rotundata Elongation Factor 1-Alpha	100878583
Megachile rotundata Glyceraldehyde-3-Phosphate Dehydrogenase 2-Like	100874769
Megachile rotundata Glutathione S-Transferase 1-1-Like	100879899
Megachile rotundata Porphobilinogen Deaminase	100878868
Megachile rotundata Eukaryotic Translation Initiation Factor 5A	100881759
Megachile rotundata Reactive Oxygen Species Modulator 1	100876106
Megachile rotundata NADH Dehydrogenase [Ubiquinone] 1 Alpha Subcomplex Subunit 8	100878306
Megachile rotundata Ruvb-Like 1)	105661911