DEVELOPMENTAL CHANGES IN FAT METABOLISM IN THE TOBACCO HORNWORM, *MANDUCA SEXTA*

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ABSTRACT

Insects fed high carbohydrate diets have increased fat storage, and excess fat storage in insects may have fitness costs. *M. sexta* larvae reared on 5.6% fat diet had lower body mass and growth rate compared to those on 3.4% fat or 0.4% fat diets. Fifth instar caterpillars fed high-fat diet ate less food but had higher approximate digestibility than the low-fat diet fed caterpillars. Analysis of fat body and fecal pellet lipid content showed high-fat diet fed caterpillars stored and excreted more lipids than low-fat diet fed caterpillars. To test the hypothesis that increased dietary fat alters lipid transport, we measured mRNA expression of apolipoproteins I and II, proteins for transporting lipids. Expression of apolipoproteins I and II did not differ with dietary fat. Negative feedback from fat intake could inhibit feeding via endocrine pathways. This research will increase our understanding of the regulation of feeding in caterpillars.

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1.1. *Manduca sexta* as a model insect

Manduca sexta, also known in its larval form as the tobacco hornworm caterpillar, belongs to the family Sphingidae, order Lepidoptera and class Insecta. In the wild, *M. sexta* feeds on plants of the family Solanaceae (e.g., tomato, potato and tobacco), and as such, has been an important crop pest. Larvae of *M. sexta* are also known to feed on non-solanaceous plants, and ovipositing female moths have been found to prefer non-solanaceous plants (Mechaber and Hildebrand, 2000). Female *M. sexta* moths release species-specific chemicals called pheromones which attract male moths to female moths during the time of mating (Stengl, 2010).

M. sexta has been reared in the laboratory since the late 1960's for use in scientific research because of its large body mass and ease of rearing on artificial diet (D'Amico et al., 2001). It has a short life cycle and is multivoltine, which means that it can have many generations each year. As such, M. sexta has become an excellent model organism for physiological, endocrinological, and immunological studies.

In laboratory conditions, *M. sexta* generally has five larval stages called instars and can grow from 1 mg in the first instar to more than 10 g during its final instar (Reinecke et al., 1980; Goodman et al., 1985). First instar larvae show higher growth rates compared to fifth instar larvae, despite their smaller mass (Reinecke et al., 1980). Interestingly, the number of larval instars is inversely correlated with growth rate in the wild, where *M. sexta* larvae with slower growth rates have six instars and those with faster growth rates have five instars (Kingsolver, 2007).

The end of a larval instar is marked by the shedding of the old exoskeleton, in a process

called molting. Molting is controlled by the coordinated actions of prothoracicotrophic hormone (PTTH), juvenile hormone (JH) and ecdysone (Bollenbacher et al., 1987). Much of the research on insect molting has focused on larval to pupal development (Bollenbacher et al., 1975; Nijhout, 1975; Nijhout and Williams, 1974; Riddiford et al., 2003). Larval to larval molting in *M. sexta* has been studied in animals at the end of the fourth instar. The larval to larval molt is initiated by the release of PTTH in a single burst, whereas in the final molt to the pupal stage in the fifth instar, PTTH is released in three bursts (Bollenbacher et al., 1987). PTTH activates prothoracic glands to release ecdysone. In the presence of juvenile hormone, ecdysone initiates a larval-to-larval molt (Nijhout and Williams, 1974, Nijhout 1981). Ecdysone in the absence of JH signals the onset of metamorphosis, during which the larvae transitions to the adult form (Nijhout and Williams, 1974). The decreased concentrations of JH and ecdysone turn off larval cuticle genes and turn on the pupal cuticle genes, preparing the larva for metamorphosis (Riddiford, 2003).

1.2. Nutritional requirements of *Manduca sexta*

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). This type of diet-mixing is displayed by grasshoppers, which feed on a variety of plant species (Iv, 1933). *Hyalophora cecropia* moths, which feed on a single plant species, also mix their diets for optimal nutrient intake by eating different parts of the plant (Scriber, 1977). An analysis of the nutritional content of *Datura innoxia*, one of the host plants of *M. sexta*, revealed that different parts of the plant had different levels of nutrients (Ayuba et al., 2010). The seeds contain 15.52% lipid and 13.9 % protein, while the stem, pod and root contain 6 % lipid and 2.9% protein. Nitrogen content also varied within a plant; the stem had 20.88% nitrogen, and the pod had 47.99% (Ayuba et al., 2010). Diet

mixing is a common mechanism for insects to obtain limiting nutrients in nature.

In the laboratory, artificial insect diets are nutritionally balanced and allow for easy manipulation of nutrients. Since Bell and Joachim (1967) 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*. Carbohydrates are required for fat synthesis, and *M.sexta* larvae reared on a low-sucrose diet deposited less fat in the body, even though 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 and Wang, 2003).

Dietary protein is also important for proper growth. Fifth instar larvae of *M. sexta* reared on a low protein diet grew slower and showed compensatory feeding, increasing food intake to obtain adequate protein (Woods, 1999; Ojeda-Avila et al., 2003). Fifth instar *M. sexta* larvae reared on a carbohydrate-free diet showed increased fat content due to higher consumption of protein (Thompson and Wang, 2003; Schilder and Warden, 2006; Warbrick-Smith et al., 2006)

Although, most studies have concentrated on organic nutrients, other nutrient elements like dietary phosphorous and nitrogen are also crucial for insect growth and development. *M. sexta* grow better on a high phosphorous diet and require about 3 mg of phosphorous per day for normal growth and development (Perkins et al., 2004). RNA, which is required for protein synthesis during growth, contains 10% phosphorous (Elser et al., 1996). Another element crucial for insect growth is nitrogen, and insects show compensatory feeding in response to low levels of nitrogen (White, 1978). Insects also show compensatory feeding when fed diets lacking any of the essential amino acids, methionine, threonine and lysine, indicating that a diet with essential amino acids is important for growth (Pesti, 2009).

Lipids, due to high energy value are critical for reproduction and metamorphosis (Hayes et al., 1992). *M. sexta* larvae, during the fifth instar, store a greater amount of lipid than proteins and carbohydrates (Hayes et al, 1992). In addition, insects also have essential fatty acids. For example, Lepidopteran insects require polyunsaturated fatty acids, such as linolenic and linoleic acids, in their diet for normal growth and formation of adult wing scales (Fraenkel and Blewett, 1946). But other insects, like cockroaches, termites, and crickets do not require linoleic acid, because they can synthesize it (Blomquist et al., 1982). In larval *M.sexta* dietary triacylglycerol is hydrolysed in the midgut to free fatty acid but transported from the midgut to fat body for storage as diacylglycerol by lipophorin (Tsuchida and Wells, 1988). When larvae of *M. sexta* were reared on fat free diet were smaller and larger fat bodies compared to those reared on control and high fat diets (Fernando-Warnakulasuriya et al., 1988).

1.3. Digestion and absorption of lipid

Herbivorous insects ingest lipid in the form of phospholipids and triglycerides (triacylglycerols, TAG), both of which must be digested before being used (Beenakers et al., 1985). Lipid is digested in the midgut of insects (Tsuchida and Wells, 1988). When the hemipteran insect, *Triatoma infestans*, was fed radio-labeled oleic acid, most of the radioactivity occurred in the midgut (Rimoldi et al., 1985). TAG is hydolysed to free fatty acids in the lumen of the midgut (Turunen, 1993; Canavoso and Wells, 2000). The midgut cells of insects have a basement membrane, which hinders lipid absorption and therefore most of the digested lipid is excreted without being absorbed by the midgut (Turunen, 1979). In contrast, in mammalian midgut cells, which lack a basement membrane, the lipid readily crosses cell membranes and is absorbed efficiently (Turunen, 1979). Lipases play an important role in the physiology of digestion in insects (Grillo et al., 2007). Phospholipase A, the major enzyme responsible for

lipid breakdown, has been found in the guts of the cabbage butterfly *Pieris brassicae*, the beet armyworm *Spodoptera triguens*, and the cabbage looper *Trichoplusia ni* (Somerville and Pockett, 1976) and in the whole body and the midgut of the larval mosquito, *Aedes aegypti* (Nor Aliza and Stanley, 1998). Phospholipase A has also been found in the salivary gland of *M. sexta* (Tunaz and Stanley, 2004), although another study found that TAG is completely hydrolyzed to free fatty acids in the midgut of *M. sexta* (Tsuchida and Wells, 1988).

Absorption of digested lipid occurs in the midgut of insects (Tsuchida and Wells, 1988). After the hydrolysis of TAG into free fatty acids an glycerol the products of digestion are absorbed by the walls of the midgut lumen (Turunen, 1993; Canavoso et al., 2004). In *Stomoxys calcitrans*, absorption of lipid takes place in the posterior region of the midgut (Lehane, 1977). In *M. sexta*, fatty acids produced by the hydrolysis of TAGs are absorbed by the midgut cells and released into the haemolymph as DAG (Tsuchida and Wells, 1988). In *Rodnius prolixus*, the posterior midgut epithelium was the major site for absorption of free fatty acids produced by the hydrolysis of TAG (Grillo, 2007). A possible mechanism for the formation of diacylglycerol is esterification of free fatty acid inside the midgut cells and its release into the haemolymph, similar to vertebrates (Weintraub and Tietz, 1973). However, Turumen and Chippendale (1977) explained the appearance of DAG in the haemolymph as being a product of the lipolysis of TAG in the gut lumen and subsequent absorption of DAG by the midgut cells. Canavoso (2004) showed that the formation of DAG from free fatty acids in hemipteran insects is via the phosphatidic acid pathway by the incorporation of labeled oleic acid into phosphatidic acid. In the cockroach, *Periplanata americana*, the presence of monoacylglycerol transferase activity was observed in the crop, midgut and fat body, and the formation of DAG from

monoacylglycerol suggests the likelihood of monoacylglycerol pathway (Hoffman and Downer, 1979; Canavoso and Wells, 2000).

1.4. Fat body and lipid storage

Insects store and synthesize fats, proteins, and glycogen in an organ called the fat body. The fat body is composed of cells that are poorly connected, and therefore it appears as a loose structure dispersed throughout the body (Law and Wells, 1989). The fat body occurs as thin lobes underneath the integument surrounding the entire abdomen and reproductive organs of insects and is bathed by haemolymph (Arrese and Soulages, 2010). Insects store nutrients in the fat body to supply energy during non-feeding periods and in the adults for reproduction and flight (Ziegler, 1991). Holometabolus insects, those that undergo complete metamorphosis, require lipid storage to provide energy for metamorphosis. *M. sexta*, during its last larval or fifth instar, increases the lipid stored in the fat body (Fernando-Warnakulasuriya et al., 1988). Ninety percent of the lipid is stored in the form of TAG in the fat body as the major energy store (Arrese et al., 2010).

The fat body is a multi-faceted organ. Cells of the fat body release antimicrobial peptides, playing a role in insect immunity (Ferrandon et al., 2007). However, the most important role of the fat body is fat storage. Other cell types found in the fat body include the urocytes, which are specialized for uric acid storage; the mycetocytes, which contain microorganisms; and the oenocytes, which are involved in cuticular protein, lipid and hydrocarbon synthesis (Arrese and Soulages, 2010). The fat body of fifth instar *M. sexta*, consists solely of trophocytes and functions as both a TAG- and urate-accumulating organ (Willott et al., 1988).

1.5. Lipophorin

In insects, as in vertebrates, lipid transport is accomplished by lipoprotein complexes (Ryan, 1990). Lipoprotein complexes are composed of lipid and protein particles, and in insects the major lipoprotein is called lipophorin. Lipophorin is a spherical particle with a hydrophobic core, consisting of hydrocarbons and DAG (Soulages and Wells, 1994). In contrast, the vertebrate lipoprotein core is dominated by TAG (Soulages and Wells, 1994). The outer layer of the insect lipophorin is composed of phospholipids and proteins. The protein is linked to the hydrocarbon chain of the phospholipid, the lipid portion is water insoluble and the protein is water soluble (Ryan, 1994).

The outer protein portion of lipophorin consists of two integral proteins, one molecule of each apolipoprotein (apo) I and II. Apo I and II are non-exchangeable proteins and do not dissociate from the lipophorin (Shapiro et al., 1984). The apoproteins have two main functions. First, they maintain the structure of the lipoprotein. Second, they reversibly bind to the plasma membrane which facilitates the lipid exchange between the target tissue and the lipophorin (Shapiro and Law 1984). Apo I is located on the outside of the lipoprotein, while apo II occupies the deeper layer of the lipophorin complex (Shapiro et al., 1984). A single precursor protein for both apo I and II is expressed in the fat body of *M. sexta,* and the protein is encoded by a 10,138 bp cDNA (Sundermeyer et al., 1996). Studies in the locust, *Locusta migratoria*, also showed that the apolipoprotein I/II is composed of a single precursor protein, which is later cleaved into apo I and apo II (Weers et al., 1993). The cleavage occurs postranslationally, and the site of cleavage was determined to be at the consensus substrate sequence for furin (Smolenaars et al., 2005). Insect apo I and II are homologous to mammalian apoB and use the same structural elements during biogenesis as its mammalian homologue (Smolenaars et al, 2007).

1.6. Lipid transfer particle

Lipophorin requires assistance from a lipid transport particle (LTP) to load and unload DAG. *In vitro* and *in vivo* studies on LTP of different insect species revealed the common property and role of LTP in catalyzing the transfer of lipids between lipophorins, lipophorin and tissues and oocytes. Isolation and characterization of LTP from the hemolymph of *M. sexta* revealed that it has a density of 1.23 g/ml and contains two large apoproteins, apo LTP-I and apo LTP-II (Ryan et al., 1986).

Once digested and absorbed, the lipid must be transported through the haemolymph foruse or storage. TAG, after hydrolysis into free fatty acid is absorbed and converted into DAG in the midgut and transported to the fat body (Canavoso et al., 2001). In the fat body, DAG is again converted into TAG for storage (Canavoso et al., 2001). Since lipids are water insoluble, a transport system is required. In insects, lipid is transported by lipoproteins called lipophorins and egg yolk protein precursor called vitellogenin (Ryan, 1990). Vitellogenin has been isolated and purified from M. sexta and is similar to high density lipoprotein of M.sexta (Pattnaik et al., 1979). In *M. sexta,* DAG is loaded on to the lipophorin from the midgut by a LTP (Canavoso and Wells, 2000) and unloaded from the lipophorin to the fat body by LTP (Canavoso al., 2003). The LTP also mediates the loading of DAG from the fat body to lipophorin (Van Heusden and Law, 1989). *In vitro* studies have shown that LTP aids in the transfer of DAG between lipophorins (Ryan et al., 1986). LTP also transfers lipid from high density lipophorin (HDLp) to vitellogenin, a protein found in the female insect haemolymph (Tsuchida et al., 1997). A similar role of LTP in the catalyzed transfer of lipids between lipophorin and oocytes was found in the haemolymph of female *Rhodnius prolixus* (Golodne et al., 2001).

The lipophorin has specific binding sites at the tissues, and lipid is transferred on the

surface of the cell without internalization (Atella et al., 1992). Different tissues have different lipid requirements. For example, cuticles require hydrocarbons, and silk glands require carotenoids, and lipophorin delivers lipids to each of these tissues (Law and Wells, 1989). From the fat body of *M. sexta*, a lipophorin receptor was isolated and found to be similar to the LDL receptor of mammals and chickens (Tsuchida and Wells, 1990). Another vertebrate homologue of low density lipoprotein receptor was isolated from the locust fat body, oocytes and midgut (Dantuma et al., 1999). The binding of lipophorin to midgut cells was studied using iodinated lipophorin. Unlike the binding of the lipophorin in the fat body, which was dependent on the DAG content of the lipophorin, the binding of lipophorin to the midgut membrane was independent of the lipid content of lipophorin (Gondim and Wells, 2000). Although the vertebrate low density lipoprotein (LDL) receptor is homologous to insect high density lipoprotein receptor or insect lipophorin (iLR), there are subtle differences that separate the insect and vertebrate lipophorin receptors. Vertebrate LDL receptor is degraded by the lysosomal enzymes, whereas the iLR ends up in a recycling compartment from where it escapes and is recycled (Dantuma et al., 1999).

1.7. Food utilization efficiency

The efficiency with which insects utilize food can be determined by calculating

1) efficiency of conversion of digested food

$$\text{ECD} = \frac{\text{weight gained by the larvae during the feeding period}}{\text{weight of food consumed-weight of feces}} \text{ x } 100$$

2) approximate digestibility

$$\text{AD} = \frac{\text{weight of food consumed-weight of feces}}{\text{weight of food consumed}} \text{ x } 100$$

3) efficiency of conversion of ingested food

$$ECI = \frac{\text{weight gained by the larvae during feeding period}}{\text{weight of food consumed}} \text{ x } 100$$

(Waldbauer, G. P. and Friedman, S. 1991). ECD measures the utilization of digested food for biomass, AD measures the amount of food consumed that was not excreted and ECI measures the conversion of ingested food into biomass (Waldbauer, G. P. and Friedman, S. 1991). These indices are related as ECI = AD x ECD. AD was higher in the fifth instar compared to adult *Locusta migratoria,* because of the allocation of energy towards somatic growth rather than reproduction (Hoekstra and Beenakkers, 1976). In fifth instar *M. sexta,* AD decreased for the first two days and remained constant for the rest of the instar, while ECD increased from day one to day four. This decrease in AD and increase in ECD were explained by decrease in metabolic rate of fifth instar *M.sexta* and utilizing most of the energy for growth (Hayes et al., 1992).

1.8. Nutrient self-selection

When offered a variety of foods, animals usually select a diet that provides a balance of nutrients. This process is called dietary self-selection (Westoby, 1974). The purpose of eating balanced nutrients is to achieve optimal nutrient intake, ensuring maximal growth and development (Simpson and Raubenheimer, 1996). Insects self select a diet that maximizes growth and development, and self selection is not only induced by imbalanced nutrients but several other factors like hemolymph trehalose level and parasitization. Larvae of the flour beetle, *Tribolium casteneum*, were reared on wheat bran, wheat endosperm, or wheat germ or a mixture of these in the ratio of 1:1:1 (Waldbauer and Bhattacharya, 1973). Larvae on the mixed diet consumed 80% germ and 20% endosperm and showed normal growth and development compared to larvae on individual wheat components (Waldbauer and Bhattacharya, 1973). In *M. sexta* dietary self-selection was influenced by blood sugar level (Thompson and Redak, 2000). Given a choice of high or low content of sucrose and casein, larval *M. sexta* with high blood

sugar level preferred a low casein diet, and larvae with low blood sugar levels preferred a sucrose diet (Thompson and Redak, 2000). Larvae preconditioned to a diet devoid of sugar preferred a sugar-rich diet when offered both sugar-rich and protein-rich diets, but this preference was absent when the same larvae were injected with trehalose (Friedman et al., 1991). Normal *M. sexta* larvae self-select a diet of 2:1 protein to carbohydrate, while parasitized larvae show altered dietary self-selection behavior, ingesting protein to carbohydrate ratios of 1:1 (Thompson et al., 2001). Parasitized larvae after preconditioning to either protein or sucrose diet did not show preference for 2:1 protein to carbohydrate ration and fed randomly compared to unparasitized larvae (Thompson et al., 2001). Food induction (preference for diet on which the larvae were reared) plays an important role in nutrient self selection (Stadler and Hanson, 1978). Larvae of *M. sexta* reared on leaves, artificial diets, or homogenized leaves mixed in the artificial diets were able to discriminate food due to food induction (preference for diet on which the larvae was reared), and they preferred food that they were conditioned (process of acclimating an insect to a given food) to in the food choice tests (Stadler and Hanson, 1978)

Waldbaeur and Friedman (1991) introduced the "malaise hypothesis" which stated that insects will eat a diet deficient in 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 requirement of the deficient nutrient (Waldbauer and Friedman, 1991). When food choices are not available to provide adequate nutrients, insects ultimately eat the nutrient-deficient food, which results in health risks related to the deficiency of that nutrient while storing the excess of unwanted nutrients (Raubenheimer, 1992). *M. sexta* larvae reared on artificial diet or natural *Datura wrightii* leaves with high levels of phosphorous had faster growth rate and shorter developmental time than the larvae on low phosphorous foods (Perkins et al., 2004). But fifth instar larvae of

M. sexta, reared on phosphorous poor leaves of *Datura wrightii*, 85% of the phosphorous was stored in the body but when phosphorous rich *Datura wrightii* leaves were fed, only 25 % was stored while 75% was excreted in the feces (Woods et al., 2002).

1.9. Regulation of food intake

In insects, food intake is regulated by the central nervous system and has been well studied in the blowfly, *Phormia regina* (Dethier and Gelperin, 1967). Feeding in these insects was initiated by the sensory stimulation of peripheral chemoreceptors and terminated by the inhibition of input from the foregut to brain (Dethier and Gelperin, 1967). The role of central nervous system in the regulation of feeding has also been studied in *M. sexta* larvae (Rowell and Simpson, 1992). When the nerve connectives in the region between the head and thorax were cut, the fifth instar larvae showed continuous chewing or feeding indicating that chewing or feeding is controlled by the central nervous system (Rowell and Simpson, 1992). Neurotransmitters, like serotonin, have also been shown to regulate specific nutrient intake in the larvae of *Heliothes zea*, where protein consumption was increased when serotonin was added to the diet (Cohen et al., 1988). Other neurotransmitters like octopamine and synephrine, though not nutrient-specific like serotonin, when added to the diet of cockroach nymph, *Rhyparobia Madera* caused increased feeding (Cohen et al., 2002). Studies on regulation of food intake has been concentrated on proteins and carbohydrates but studies on regulation of nutrient intake like the lipid is lacking.

1.10. Conclusion

Using insects as model systems to study fat metabolism and regulation of feeding have provided insights into underlying mechanisms. There is a lot of information regarding fat digestion, absorption and transport in insects, but it is unclear how these are affected by high fat

diets. There is very little knowledge about how insects regulate food intake and studies have been focused on protein and carbohydrates but how do they regulate high lipid intake is still not clear. As reviewed in this study many of the pathways involved in fat metabolism of insects are similar to vertebrates which make insects ideal for studies of the effects of high-fat diet and also help answer many questions related to high-fat diet related human health problems.

CHAPTER 2: DEVELOPMENTAL CHANGES IN FAT METABOLISM IN TOBACCO HORNWORM, *MANDUCA SEXTA*

2.1. Introduction

Many herbivorous insects require lipids in their diet for normal growth and development (Sivapalan and Gnanapragasam, 1979). In the sphingid moth *M. sexta*, dietary fat serves as an energy source for metamorphosis, adult emergence, flight, and reproduction. In *M. sexta,* the lipid storage organ, or fat body, changes its role from lipid storage during the larval and pupal periods to lipid mobilization during the pupal and adult stages (Patel et al., 2005).

In larval insects, lipids are digested and absorbed in the midgut and then transported to the fat body for storage. The lipid component of dietary fat consists of triacylglycerol (TAG) which is hydrolyzed in the midgut lumen by TAG-lipase and converted into diacylglycerol (DAG; Grillo et al., 2007). During larval stages in *M. sexta,* DAG is transported from the midgut through the haemolymph to the fat body by lipophorin, the major insect lipoprotein. Lipophorin is a high density lipoprotein (HDL), composed of the lipid being transported and two very large apoproteins, apolipoprotein I and II (MW: 250, and 80, kDa) (Shapiro et al., 1984). With the help of a lipid transfer particle, lipophorin takes up DAG from the midgut and delivers it to the fat body, where it undergoes esterification by DAG acyltransferase and is then converted back to TAG, the major lipid component stored in fat body (Arrese and Soulages, 2010**)**.

Diets with nutrient imbalances can have metabolic costs, causing reduced growth, survival, and reproduction (Boersma and Elser, 2006). For example, two species of grasshopper, *Locusta migratoria* and *Schistocerca gregraia,* fed diets with excess protein or carbohydrate during the first three days of their last larval stages showed decreased fitness (Simpson and Raubenheimer, 2004). With excess protein, mortality rates were higher, but development was

faster, whereas survival increased on an excess carbohydrate diet (Simpson and Raubenheimer, 2004). Higher dietary phosphorus content (3.5% P) resulted in reduced growth rate in mayflies, *Ephemerella sp*, and larval *M. sexta* compared to food with 1% P (Frost and Elser, 2002; Perkins et al., 2004).

In addition to reductions in growth, alterations in energy storage may occur. *M. sexta* larvae reared on low protein diets (protein to carbohydrate ratio of 20:38) deposited more fat in the body and showed slower growth in the fifth instar (Ojeda- Avila et al., 2003). Larval *Plutella xylostella*, reared for one generation on a diet with high carbohydrate showed increased fat storage (Warbrick-Smith et al., 2006). The fat content of *M. sexta* increased with increasing levels of carbohydrate in the diet (Thompson and Wang, 2003). These studies indicate that insects deposit high amounts of fat with increased consumption of dietary carbohydrate and fat, similar to the results when vertebrates ingest excess calories.

While several studies have documented increases in fat storage as a result of excess carbohydrate ingestion, only a handful of studies have investigated the effect of manipulating dietary fat in insects. Fernando-Warnakulasuriya *et al*. (1988) studied the effects of dietary lipid on larval development and fat storage of *M. sexta*. Larvae on control and high fat diet had similar body masses at the end of the fifth instar. Yet, development was negatively affected by the high fat diet, as the high-fat diet delayed development by one day compared to control caterpillars (Fernando-Warnakulasuriya and Wells, 1988). The size of the fat body and fat content of caterpillars on high-fat diet were higher than those on control and fat-free diets (Fernando-Warnakulasuriya and Wells, 1988). However, the study on effects of high-fat diet is limited to the oldest instar and information on food consumption and food digestibility is lacking. When fruitflies, *Drosophila melanogaster* were fed high fat diets, they accumulated increased levels of

fat in cardiac muscles leading to heart dysfunction (Birse et al., 2010). It also affected their flight activity as the high-fat diet fed flies remained at the bottom of the vial compared to normal-diet fed flies (Birse et al., 2010).

Recent studies on insects have revealed that insects can show symptoms of obesity during experimental manipulation. The first study on insect obesity was done by Schilder and Marden (2006) using parasitized dragonflies. These flies were considered obese because they accumulated fat in the thorax region similar to symptoms of obesity observed in humans and had an adverse effect on flight activity (Schilder and Marden, 2006). In the *Drosophila* study described above, flies showed symptoms of obesity indicated by the accumulation of fat in the nonadipose tissue, similar to that observed in humans (Birse et al., 2010). Activity level of high fat diet-induced obese flies also decreased compared to flies that were not fed high fat diet (Birse et al., 2010).

Therefore, in this study, we aimed to determine the effects of a high fat diet on developing tobacco hornworms, *M. sexta*. Because of the importance of fat storage during the larval stages, we hypothesized that increasing dietary lipid affects fat deposition, growth and development of *M. sexta* larvae. We predicted that increased dietary fat would result in increased body size and fat storage. We reared *M. sexta* on high, medium or low fat diets and recorded their growth and development. Instead of increasing body size, *M. sexta* on high fat diets were smaller and grew more slowly than caterpillars on the other diets, so we investigated the mechanism underlying the decrease in body mass and development rate. Because fifth instar *M. sexta* are more efficient in digesting and absorbing food than younger instars (Gibellato and Chamberlin, 1994) and store more lipid for metamorphosis (Hayes et al., 1992), we focused the rest of our experiments on fifth instar larvae. To understand why growth was delayed on high fat

diet, we measured food consumption to test the hypothesis that the decrease in body mass and growth rate was due to a decrease in food consumption and digestibility. To further investigate the cause of lower body mass and growth rate of fifth instar *M. sexta*, we tested the hypothesis that high-fat diet-fed caterpillars store and excrete less fat. Finally, we tested the hypothesis that fat transport is altered in animals fed high dietary lipid, by measuring RNA expression of key lipid transport proteins, Apolipoproteins I and II.

2.2. Methods and materials

2.2.1. *Animal rearing*

Larvae of *M. sexta* were reared from eggs obtained either from our colony or from Carolina Biological Supply (Burlington, North Carolina). For our experiment we used larvae from our colony only to avoid variation. Hatched larvae were given *ad libitum* access to an artificial wheat germ-based diet modified from Ojeda-Avilla *et al.* (2003); (Table 1) and maintained under 16L:8D h at 25^{o} C.

Artificial diets were prepared with three levels of dietary fat, high fat (HF), medium fat (MF), and low fat (LF), levels based on previous research and was non isocaloric diet (Fernando-Warnakulauriya et al., 1988). Variation in lipid content was achieved by making the diet with different volumes of linseed oil (Table 1; HF = 5.6%, MF = 3.4%, and LF = 0.4% linseed oil by volume), as previously done (Fernando-Warnakulasuriya et al. 1988). The LF diet is the standard artificial diet fed to *M. sexta* (Ojeda et al., 2003)). Moisture content of the food was adjusted with different volumes of water.

Table 1. Ingredients of artificial diets.

Ingredient	HF	MF	LF
Wheat germ, g	80g	80 g	80 g
Casein, g	32 g	32g	32 g
Wesson's salt, g	16 g	16 g	16 g
Torula yeast, g	16 g	16 g	16 g
Cholesterol, g	3.5 g	3.5 g	3.5 g
Sorbic acid, g	2 g	2 g	2 g
Methyl paraben, g	1 g	1 g	1 g
Ascorbic acid, g	5 g	5 g	5 g
Streptomycin, g	0.2 g	0.2 g	0.2 g
Kenamycin, g	0.053 g	0.053 g	0.053 g
Vitamin mixture, ml	10 ml	10 ml	10 ml
Raw linseed oil, ml	30 ml	17 ml	4 ml
Tap water, ml	700 ml	720 ml	733 ml

The final volume of the high fat and medium fat food was adjusted with water. Each diet consisted of 1.833 % of formalin.

2.2.2. *Developmental effects of high fat diet*

First instar larvae were fed LF, MF, or HF diets right after hatching whereas second, third and fourth instar larvae were reared on LF food until they reached either the second (n =7 per treatment), fourth (n =8 per treatment) or fifth instar (n =10 per treatment), at which time caterpillars were switched to one of the experimental diets. To determine the developmental effects of high fat diet we compared the body masses and growth rates of first, second, fourth

and fifth instar larvae.

2.2.3. *Food consumption*

Fifth instar caterpillars store energy to prepare themselves for metamorphosis and therefore we measured the amount of food consumed by the fifth instar. First day, fifth instar, *M. sexta* larvae (n = 10 per treatment) were given 6 g of LF, MF or HF diet. Caterpillars always had excess food, consuming on average 50 % of the food given. Remaining food was weighed the next day, and caterpillars were given a fresh, 6 g block of food. Water loss from food was accounted for by weighing a 6 g block of food after 24 hours. To calculate food consumption, we subtracted the average water loss (0.06 ± S.E.M. g) from the amount of food consumed. This estimate of water loss is conservative, since the rate of water loss from remaining food will increase as caterpillars ingest food.

2.2.4. *Apolipoprotein mRNA expression*

Newly molted fifth instar caterpillars were given a block of LF, MF, or HF diet (n = 9 per treatment). Three caterpillars from each diet treatment were dissected for fat body mRNA extraction two and three days later and stored in -80^{o}C. Total RNA was extracted from the fat body of *M. sexta* 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), and the

reaction was carried out in a total volume of 20 µl with 1x Fast Sybergreen 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 the primer using the sequence for APO I and II (Gen Bank Accession number U57651) and online software Gen Fisher 2. To calculate absolute copy number of APO I and II, we generated a standard curve with serial dilutions of (10^8) APO I and II.

2.2.5. *Lipid content of fat body and fecal pellets*

Newly molted fifth instar caterpillars were placed with LF, MF or HF diet (n = 10 per treatment). Fecal pellets were collected daily within a period of 24 hours, until the fourth day of the fifth instar. On that day, we extracted fat bodies from caterpillars. Lipid content of both fecal pellets and fat body were determined gravimetrically using modified Folch's method for lipid extraction (Folch, J., Lees, M. and Stanley, G. H. S. 1957). We standardized the assay between samples by using the same amount of tissue or fecal pellet for lipid extraction for each individual 0.1 g for fat body tissue and 0.15 g of each fecal pellet sample. Water was removed and quantified 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 allowed to evaporate for 48 hours. The samples were then 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.

2.2.6. *Approximate digestibility (%)*

Fifth instar caterpillars (n = 10 for each treatment) were given 7 g of HF, MF or LF food

daily for each of four days. After 24 h the leftover food was weighed and transferred to a preweighed 42 ml glass vial and then dried overnight in an oven at 56^0 C. It took 5 days for the fat body and 6 days for food to get the constant dry weight. Also, 7 g of each HF, MF and LF fat diet were dried along with the leftover food. Once dried, the 7 g aliquots were weighed again. We calculated approximate digestibility using the following equation

$$AD\ (\%) = 100\ (E - F) / E$$

Where E = average dry weight of food eaten from each individual and F = average dry weight of feces produced for each diet (Reynolds, 1985).

2.2.7. *Data analysis*

IBM SPSS version 19 (SPSS Inc. 2010) was used for statistical analysis. Differences between body mass, growth rate and food consumption among treatment and control groups were analyzed by repeated measures of analysis of variance (RM-ANOVA). Two-way ANOVA was used for the analysis of mRNA expression of apolipoprotein. 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.

2.3. Results

2.3.1. *Developmental effects of dietary fat*

When experimental diets were given at first second or fourth instar, body mass (Fig. 1A; first instar: $F_{2, 15}=16.17$ $P < 0.001$, Fig. 2A; second instar: $F_{2, 21}=13.63$ $P < 0.001$, Fig. 3A; fourth instar: $F_{2, 26}=5.39$ $P < 0.025$,) and growth rates (Fig. 1A; First instar: $F_{2, 26}=5.39$ $P<0.001$, Fig. 2A; second instar: $F_{2,21}=41.34$ $P<0.001$, Fig. 3A; fourth instar: $F_{2,26}=5.52$ $P < 0.025$) of HF-fed diet caterpillars were significantly lower than that of the MF and LF diet fed larvae.

Because fifth instar larvae accumulate fats in preparation for metamorphosis, we reasoned that they would be more tolerant of a HF diet, so the rest of the experiments focused on larvae in the fifth instar. HF diet fed fifth instar caterpillars also had 50% lower body mass compared to LF-fed diet caterpillars LF (Fig. 4A; $F_{2,27} = 18.81$, $P<0.001$; Bonferroni post hoc test, $P<0.025$). Daily growth rates of caterpillars on HF diets varied differently by day compared to MF and LF diet caterpillars (Fig. 4B; $F_{4, 52} = 8.13$, $p<0.001$). Since the caterpillars varied in body mass, we corrected parameters for mass. Mass-specific daily growth rates of caterpillars also varied differently by day depending on the diet (Fig. 5; $F_{2, 26}=3.94$, $P<0.025$). Mass-specific daily growth rates of HF-fed diet fifth instar caterpillars were 80% less than the LF diet fed caterpillars by day 3 (Fig. 5; Bonferroni post hoc test, $P<0.001$). Development time was not affected by diet, as the cumulative days to wandering were not significantly different among the three diet treatments of fourth and fifth instar caterpillars (Fig. 6A; fourth instar: $F_{2,25}=1.53$ $P>0.5$, Fig. 6B; fifth instar: $F_{2,31}=0.78$ $P>0.5$).

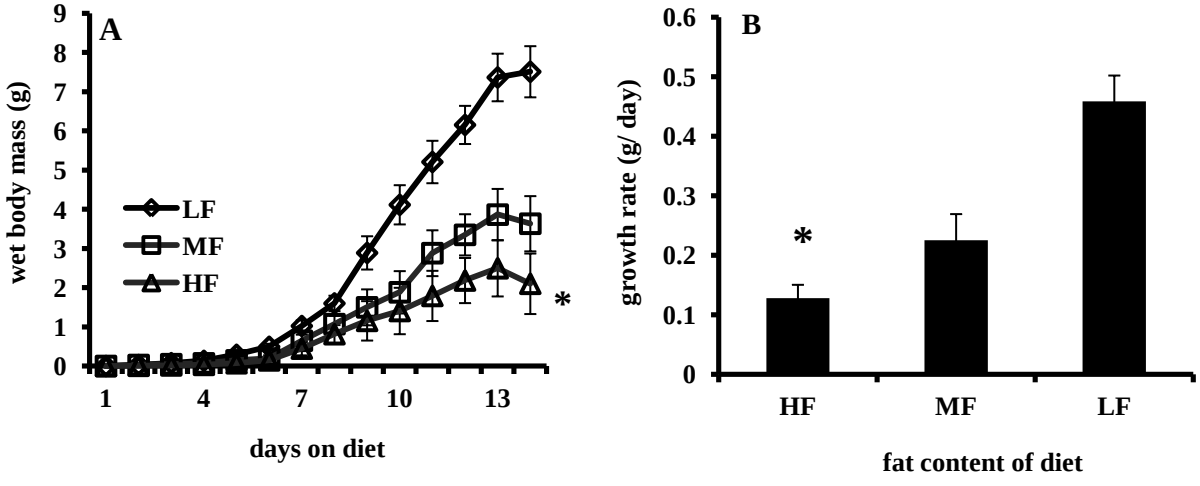

Fig. 1 A. Body mass and **B.** growth rate of first instar *M. sexta* fed high fat, medium fat or low fat diet from the first day of first instar. Asterisks represent significant differences between HF-fed animals and LF/MF animals.

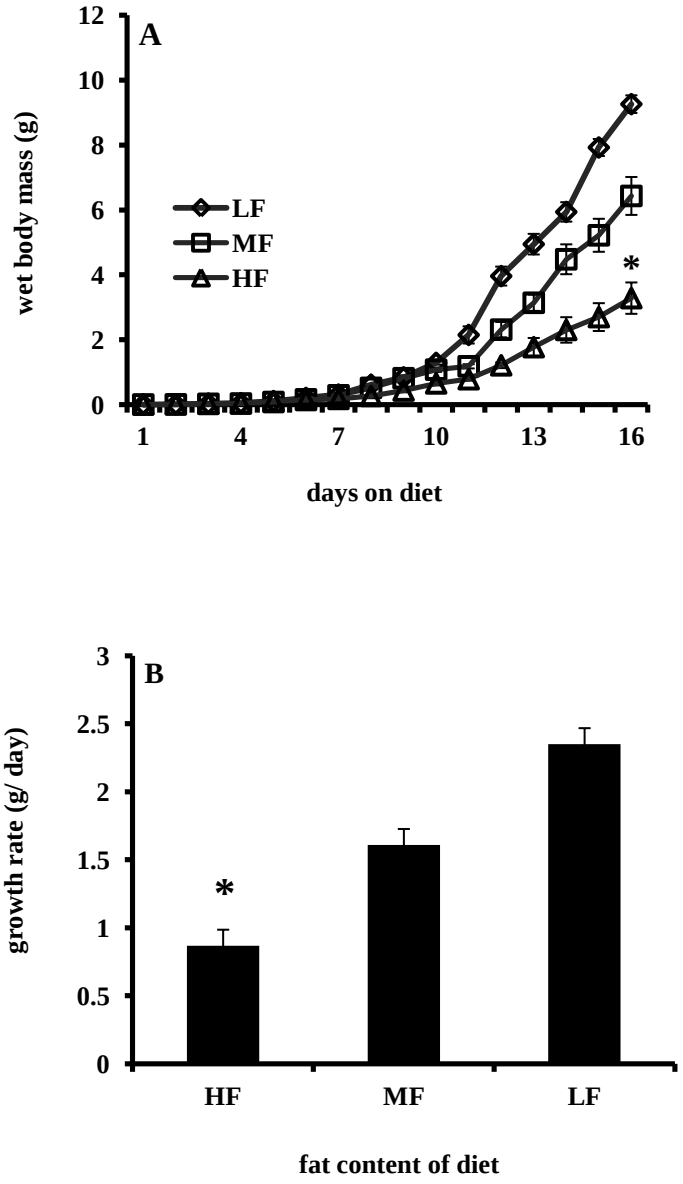

Fig. 2 A. Body mass and **B.** growth rate of second *M. sexta* fed high fat, medium fat or low fat diets from the first day of second instar. Asterisks represent significant differences between HF-fed animals and LF/MF animals.

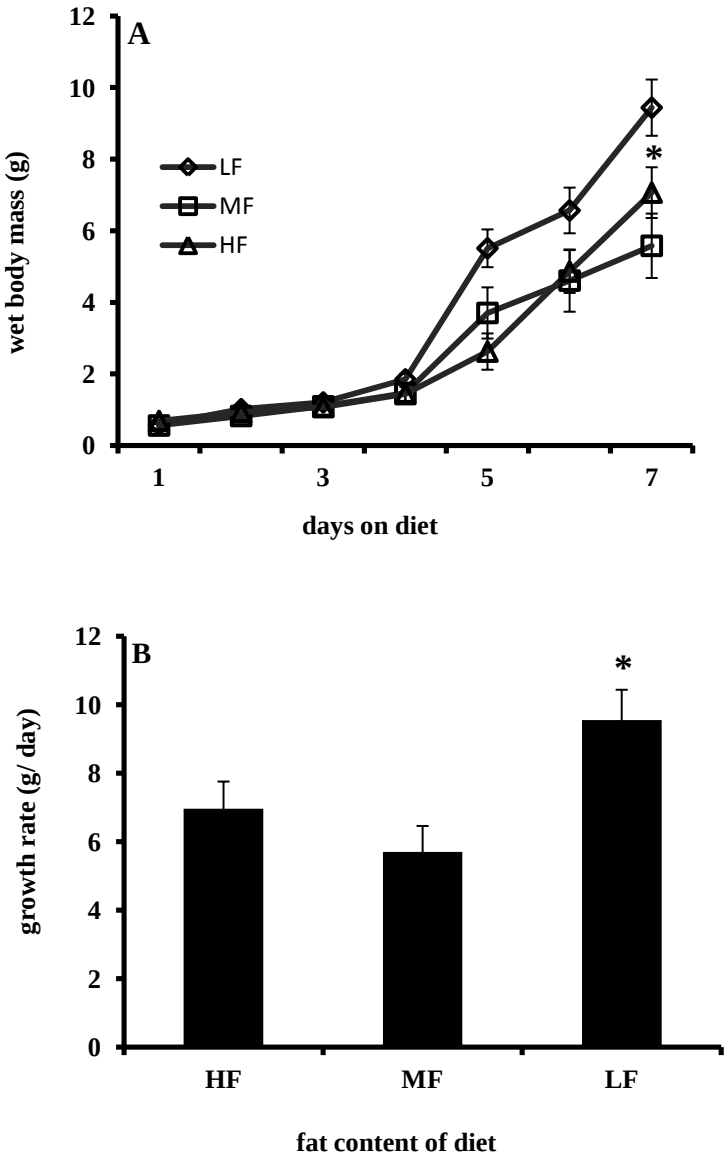

Fig. 3 A. Body mass and **B.** growth rate of fourth instar *M. sexta* fed high fat, medium fat or low fat diets from the first day of fourth instar. Asterisks represent significant differences between HF-fed animals and LF/MF animals.

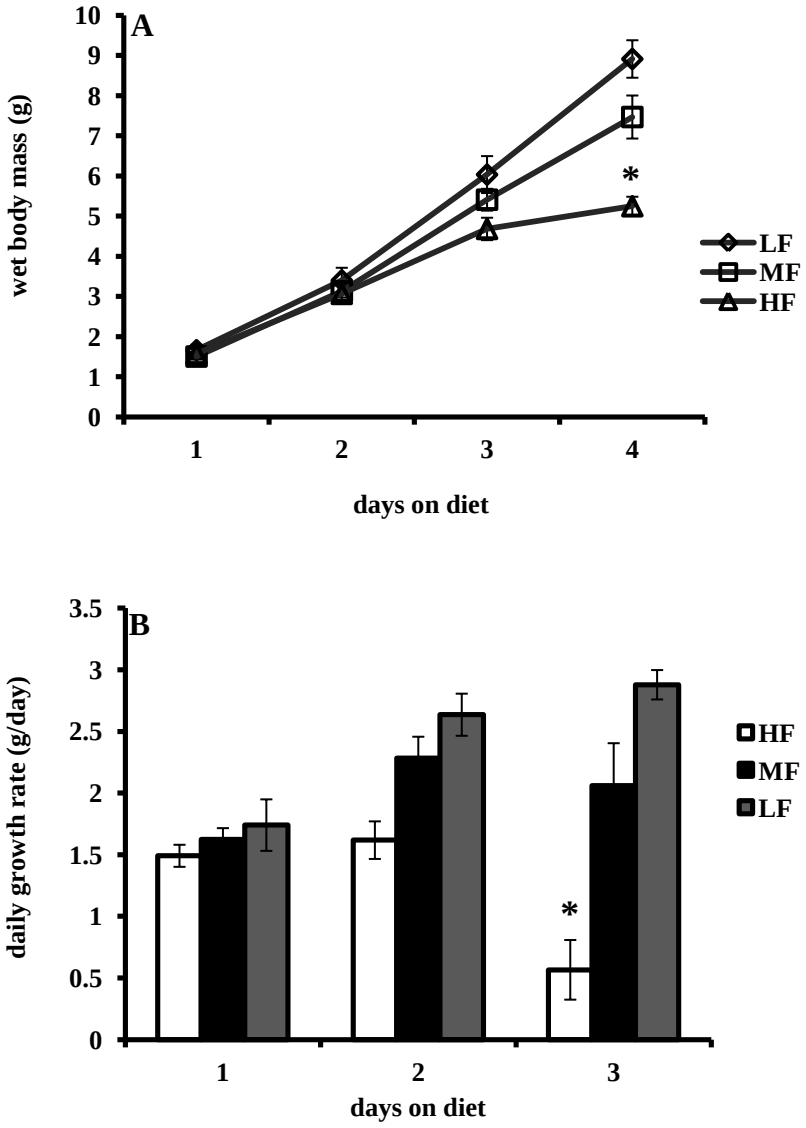

Fig. 4 A. Body mass and **B.** daily growth rate of fifth instar *M. sexta* fed high fat, medium fat or low fat diets from the first day of fifth instar. Asterisks represent significant differences between HF-fed animals and LF/MF animals.

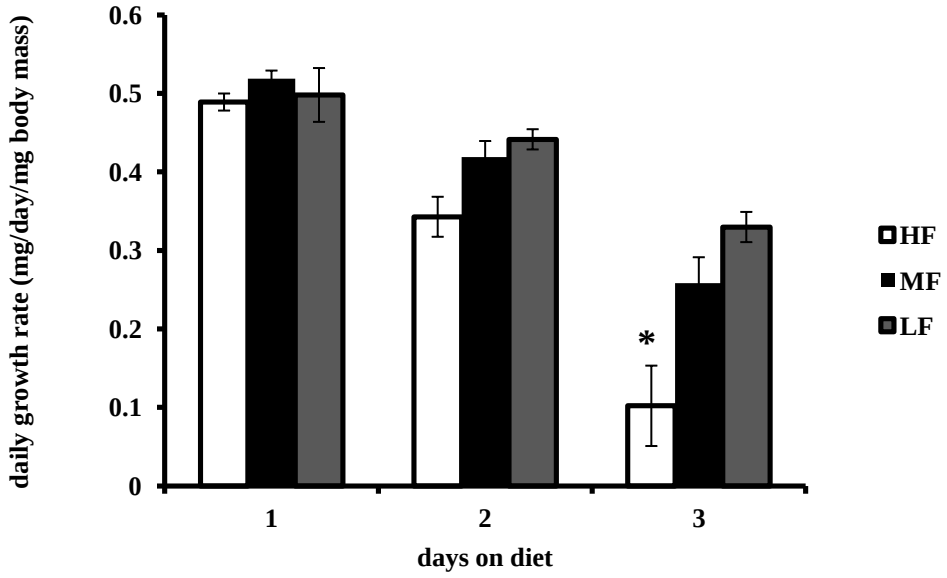

Fig. 5 Mass-specific growth rate of fifth instar *M.sexta* fed a high fat, medium fat or low fat diet. Asterisks represent significant difference between growth rates of caterpillars on HF and LF diets.

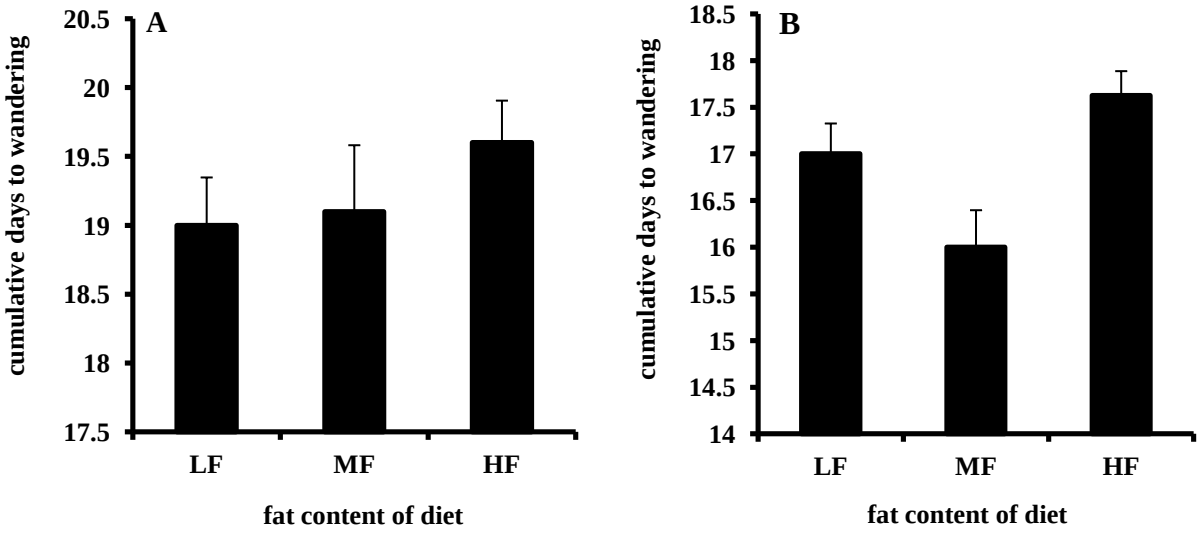

Fig. 6 Cumulative days to wandering of **A.** fourth instar and **B.** fifth instar caterpillars fed high, medium or low fat diets.

2.3.2. *Food consumption*

Because fifth instar HF-fed diet caterpillars had lower body masses than those reared on different 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 intake (Fig. 7A; $F_{2, 27}$= 8.32 P < 0.01). By the third day on the diet, HF-fed diet caterpillars consumed a third less food than LF-fed diet caterpillars (Fig. 7A; Bonferroni post hoc test P<0.025). There was no effect of dietary lipid on the mass-specific food consumption (Fig. 7B; $F_{2,27}$=0.159 P > 0.05). However, by the third day of fifth instar, mass-specific food consumption of HF-fed caterpillars was less than 50 % of the food consumed by caterpillars on the other two diets (Fig. 7B; Bonferroni post hoc test P < 0.001)

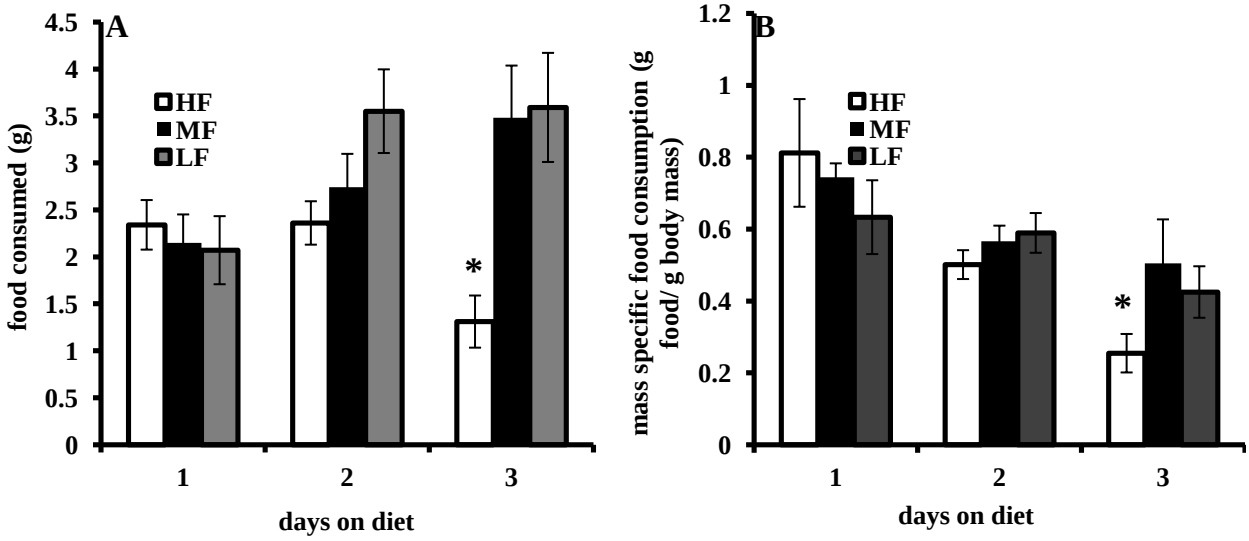

Fig. 7 A. Daily food consumption of fifth instar *M. sexta* reared on a high fat, medium fat or low fat diet. **B**. Mass-specific food consumption by day of the fifth instar for animals fed on a high fat, medium fat or low fat diet. Asterisks represent significant differences between HF and LF diet fed caterpillars.

2.3.3. *Apolipoprotein mRNA expression*

To determine whether the slowed growth was due to defects in lipid transport, we measured mRNA expression of apolipoproteins. Neither whole body expression (data not shown) nor mass-specific expression of apo varied with dietary fat content (Fig. 8).

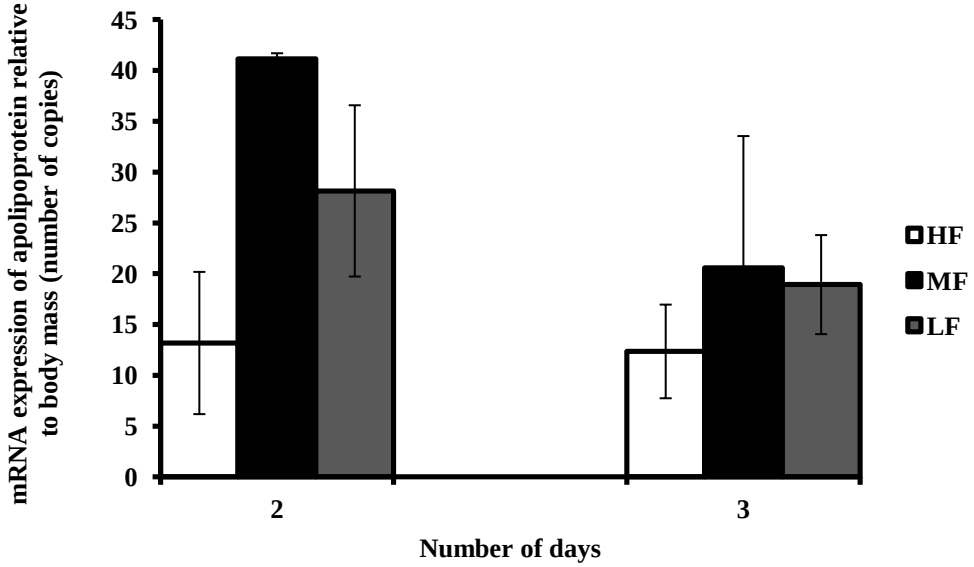

Fig. 8 Apolipoproteins I & II mRNA expression relative to body mass of *M. sexta* on the second and third day of the fifth instar after feeding on either HF, MF and LF diet.

2.3.4. *Lipid content of fecal pellets and fat bodies*

Since there was no obvious defect in lipid transport, we investigated fat storage and excretion. Absolute fat body sizes of caterpillars on different levels of dietary fat were different (Fig. 9B; dry mass: $F_{2,27}$=17.3 $P < 0.001$, Fig. 9A; wet mass: $F_{2,27}$=17.8 $P < 0.001$). Average wet mass of fat body from HF-fed diet caterpillars was eight times lower than LF-fed diet caterpillars (Fig. 9A; Bonferroni post hoc test $P < 0.001$). Dry mass of HF-fed diet caterpillars were also lower than LF-fed diet caterpillars (Fig. 9B; Bonferroni post hoc test $P < 0.025$). Because fifth instar caterpillars reared on different diets had different body masses, we corrected the wet and

dry masses of the fat bodies to body mass. Dry mass, but not wet mass, of the fat body relative to whole body mass varied with diet (Fig. 10B; dry mass: $F_{2, 27}$=4.55 P < 0.025; Fig. 10A; wet mass: $F_{2, 27}$=3.17 P > 0.05). HF-fed diet fifth instar caterpillars had almost 50% less dry weight of fat body relative to body mass compared to LF -fed diet caterpillars (Fig. 10B; Bonferroni post hoc test P < 0.03). However, there was no difference in the mass-corrected dry mass of the fat body between the MF and LF fed diet caterpillars (Bonferroni post hoc test P>0.05). Lipid content of fat body relative to whole body mass did not vary with the different levels of dietary fat (Fig. 11A; $F_{2,28}$=2.47 P>0.05). Dietary fat content significantly affected the lipid content of the fat body relative to the wet mass of the fat body (Fig. 11B; $F_{2, 28}$=4.53, P<0.025). The HF-fed diet caterpillars stored nearly three times the amount fat in their fat body compared to LF-fed caterpillars (Fig. 11B; Bonferroni post hoc test P<0.025).

The number of fecal pellets collected on the second, third, and fourth day of the fifth instar varied with dietary fat content (Fig. 12C; effect of day: $F_{1,12}$=11.47 P<0.025, effect of diet: $F_{1,12}$=6.41 P<0.025). The dry and wet mass of fecal pellet of HF-fed diet caterpillars were different (Fig. 12B, 12A; dry mass: $F_{2,12}$ = 6.4 P = 0.013, wet mass: $F_{2,12}$=12.7 P < 0.01). Mass-specific wet mass of fecal pellets varied with dietary fat (Fig. 13A; $F_{1, 27}$=5.20 p<0.035) and days ($F_{2, 27}$=4.61 P<0.025). Caterpillars on HF diet had 77% less wet mass of fecal pellet on the fourth day compared to medium and LF diet fed caterpillars (Fig. 13A; Bonferroni post hoc test, P<0.025), but there was no significant difference between the medium and LF-fed diet caterpillars (Bonferroni post hoc test, P>0.05). Mass-specific dry mass of fecal pellet decreased with the number of days (Fig. 13B $F_{1, 27}$=14.81 P<0.002), but there was no effect of dietary lipid ($F_{2, 27}$=1.15 P>0.05). Mass-specific lipid content of fecal pellets varied differently by day depending on which diet animals received (Fig. 14A; effect of day: $F_{2, 12}$=4.50 P<0.04, effect of

diet: $F_{1,12}$=6.36 P<0.03). HF diet fed caterpillars excreted nearly 50 % more lipid than LF diet fed caterpillars on the fourth day of fifth instar (Fig. 14A Bonferroni post hoc test P<0.025). Lipid content of fecal pellet per gram of fecal pellet of caterpillars on high-fat diet were also higher than those on low-fat diet (Fig. 14B; $F_{2,12}$ = 4.76 P = 0.03).

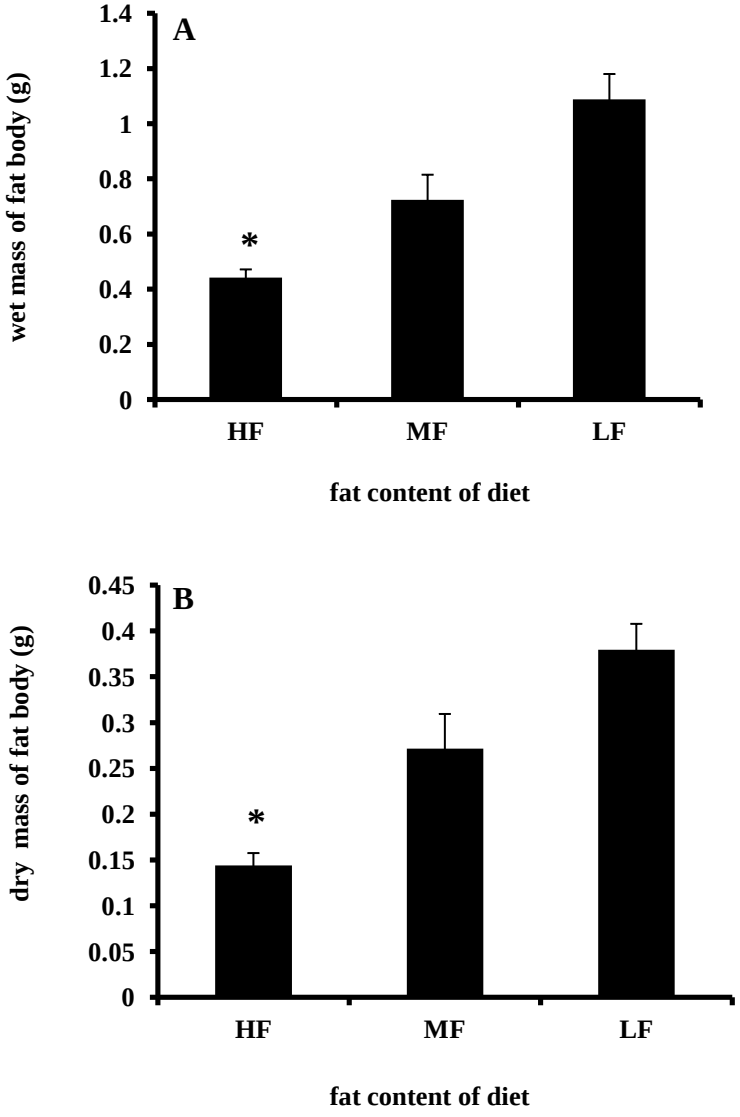

Fig. 9 A. Wet and **B.** dry masses of fat body of fith instar *M. sexta* on HF, MF and LF diets. Asterisks indicate a significant difference between HF and LF diet fed caterpillars.

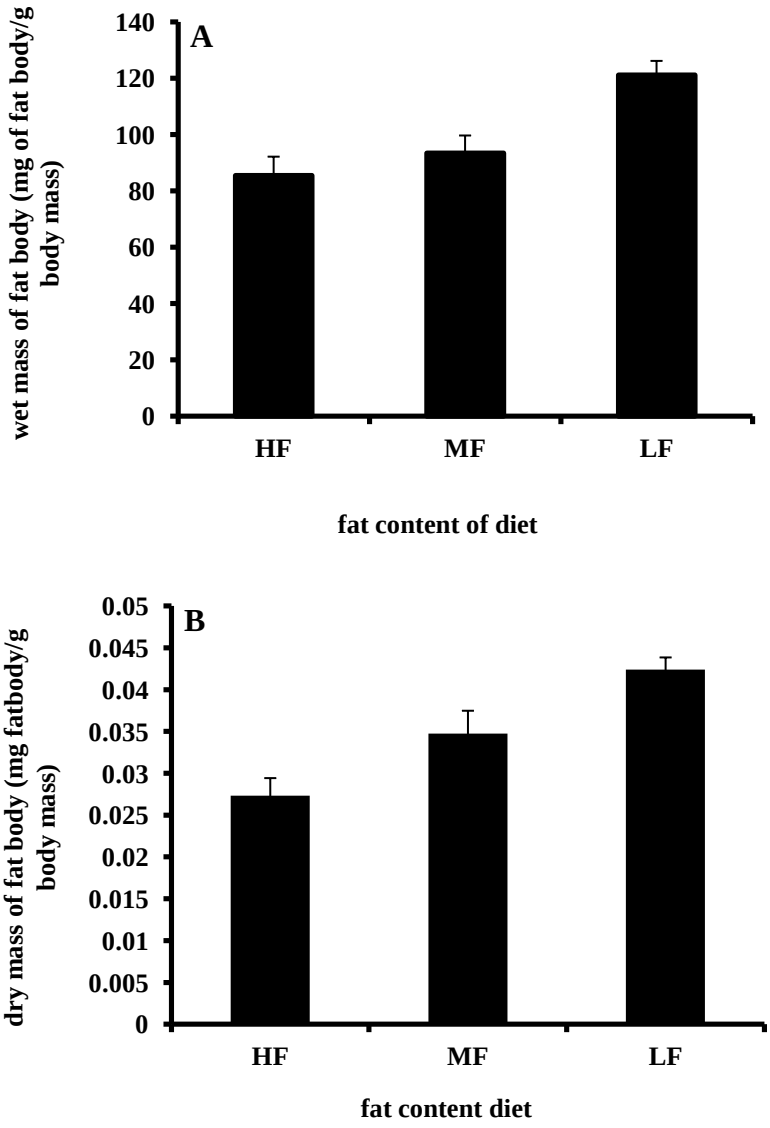

Fig. 10 Effects of eating HF, MF and LF diets on fifth instar *M. sexta* fat body on **A.** wet mass of fat body relative to body mass and **B.** dry mass relative to body mass.

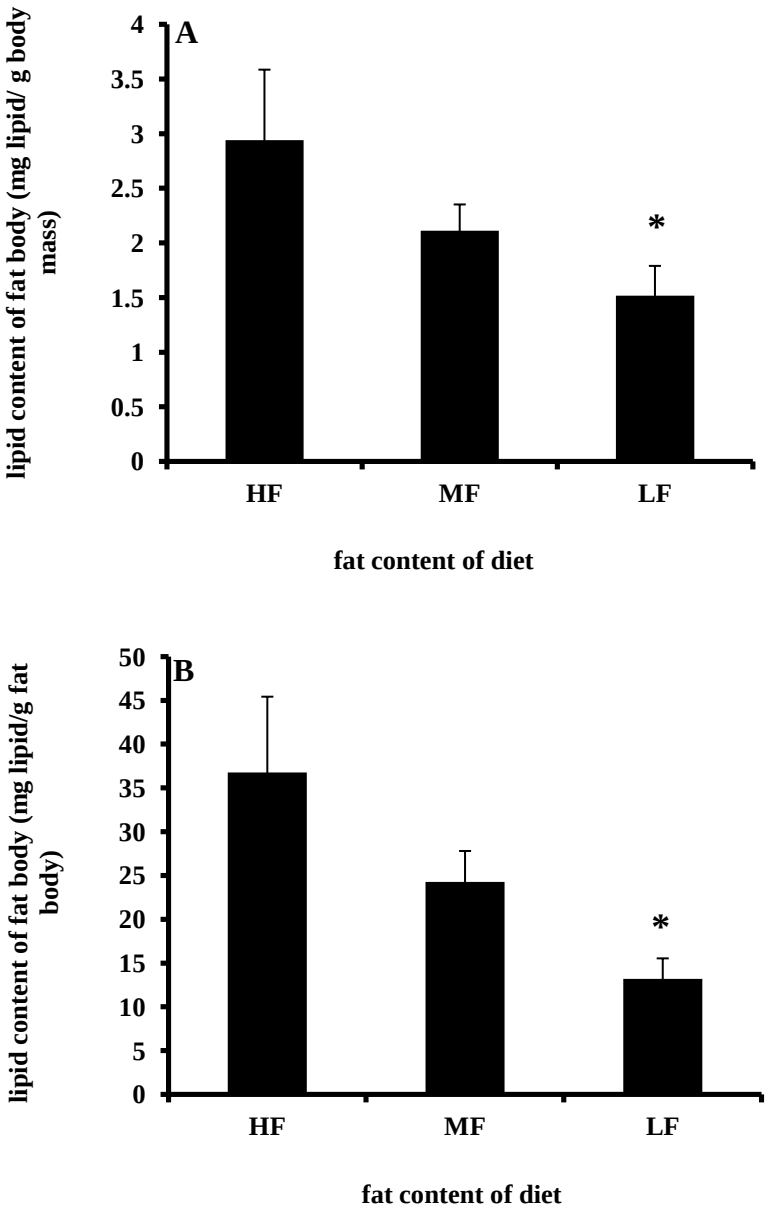

Fig. 11 Effects of eating HF, MF, and LF diets on fifth instar *M. sexta* fat body on **A.** lipid content of fat body relative to body mass and **B.** lipid content of fat body relative to wet mass of fat body. Asterisks indicate HF diet is significantly different from LF diet.

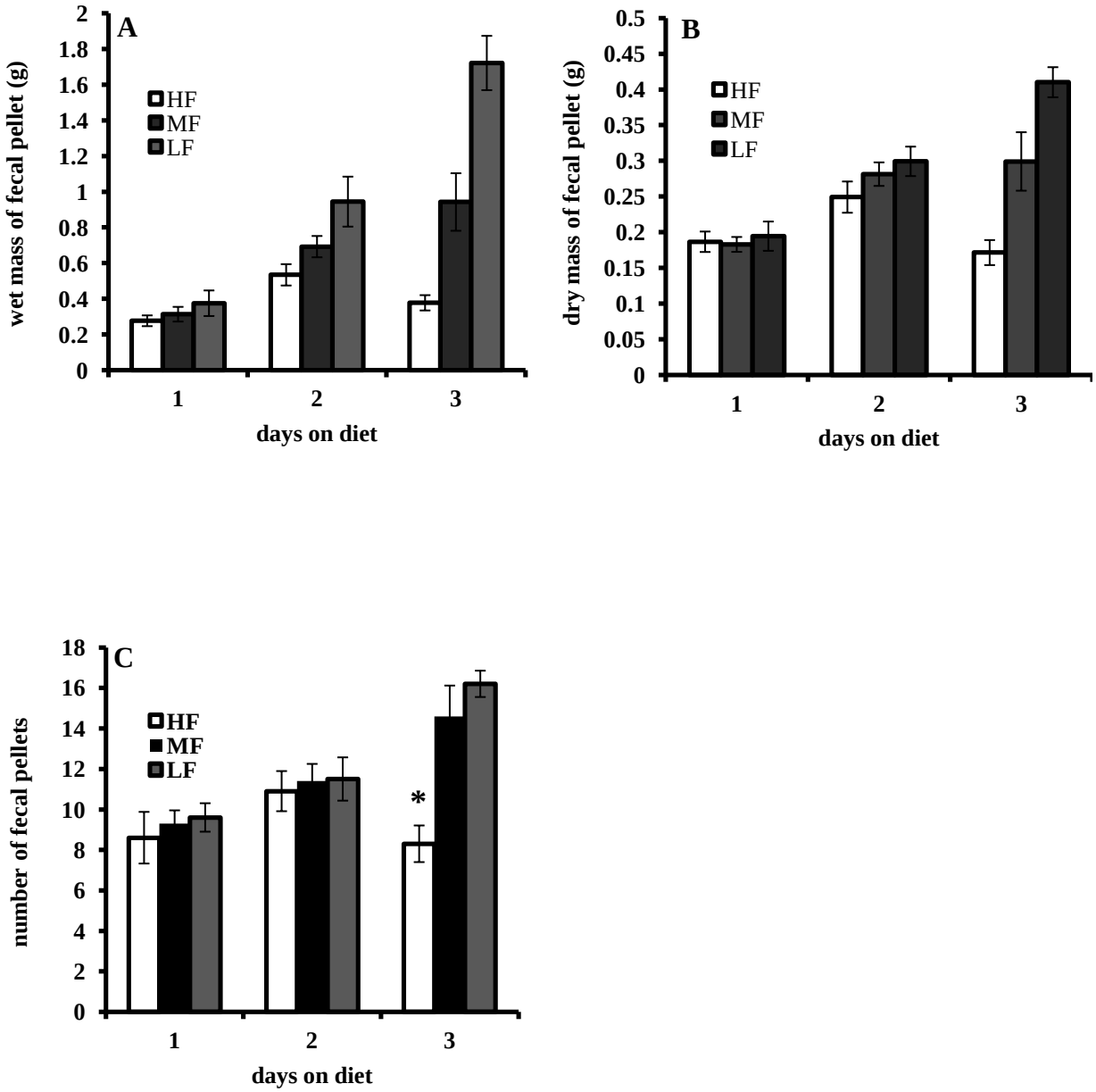

Fig. 12 A. Wet and **B.** dry mass of fecal pellets of fifth instar *M.sexta* fed high fat, medium fat and low fat diet. **C.** Nnumber of fecal pellets per day. Asterisk represent significant difference between HF and LF diet caterpillars.

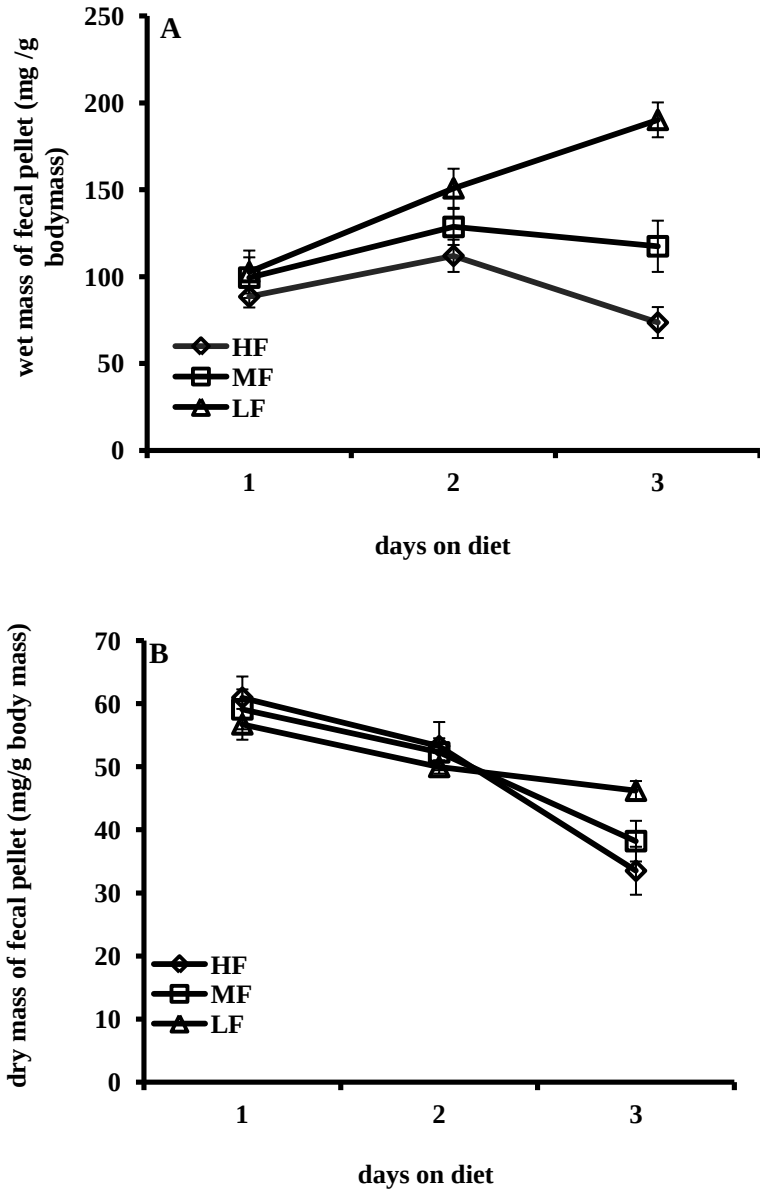

Fig. 13 Mass-specific **A.** wet and **B.** dry masses of fecal pellet of fifth instar *M. sexta* fed high fat, medium and low fat diets. Asterisk represents significant difference between HF and LF diet caterpillars.

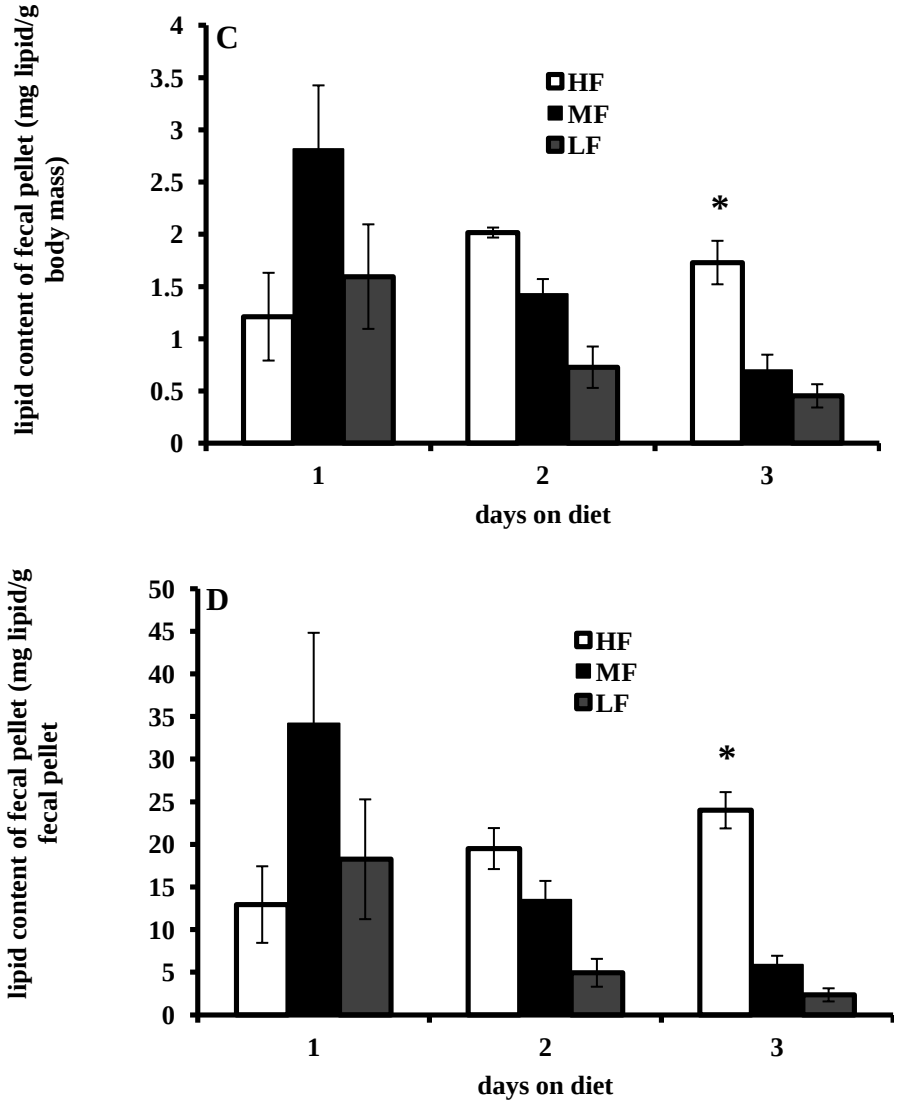

Fig. 14 Lipid content of fecal pellet of fifth instar *M. sexta* fed high fat, medium and low fat diet **A**. relative to body mass and **B.** relative to wet mass of fecal pellet. Asterisk represents significant difference between HF and LF diet caterpillars.

2.3.5. *Approximate digestibility (%)*

To further examine the decreased body mass of HF-fed caterpillars, we calculated the approximate digestibility (AD) of the three diets. AD of fifth instar caterpillars increased with the number of days into the fifth instar (Fig. 15; effect of day: $F_{1,25}=4.42$, $P<0.05$) and with HF

dietary fat content (effect of diet: $F_{2,25}$=1.77, P < 0.01). AD for HF-fed caterpillars was about 10% higher than MF or LF-fed caterpillars, until day three. On day three, AD of the LF-fed caterpillars increased to a level comparable to HF-fed caterpillars (Fig. 15, Bonferroni post hoc test, P <0.025)

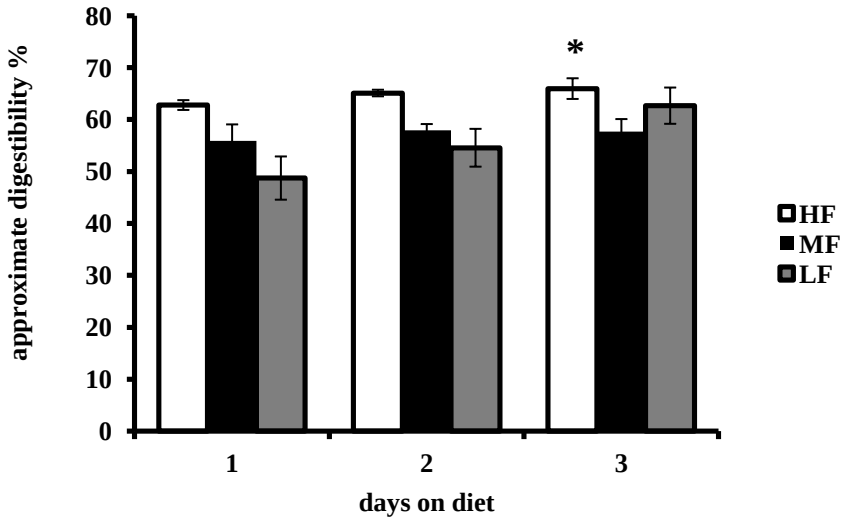

Fig. 15 Percentage of approximate digestibility per day of fifth instar *M. sexta* fed high fat, medium fat and low fat diet. Asterisk represents significant difference between HF and LF diet caterpillars.

2.4. Discussion

Our research findings were similar to the findings of Fernando-Warnakulasuriya et al., 1988, fifth instar caterpillars had higher fat content in their fat bodies. In contrast, however, we found that variation in dietary lipid negatively affects growth at many developmental stages. Caterpillars fed a high lipid diet were smaller and had slower growth than caterpillars on control diets. One study using fifth instar *M. sexta* showed that a high lipid diet caused higher deposition of fat in the fat body and increased lipid content of lipophorin **(**Fernando-Warnakulasuriya et al., 1988).

However, the negative effects on growth were surprising and in contrast to the findings of Fernando-Warnakulasuriya, Tsuchida and Wells (1988), in which their HF fed caterpillars, were as big as those fed the control diet. Our findings showed that caterpillars on high fat diet were much smaller compared to those reared on low fat diet which was our control group. Since, our data showed that caterpillars on low fat diet grew enormously but high fat diet caterpillars had lower body mass and growth rates, we speculated that high fat diet caterpillars ate less food than the low fat diet caterpillars. By the third day of fifth instar, caterpillars on high-fat diet ate less food than low-fat diet-fed caterpillars, suggesting that there was some signal to stop eating. Nicotine naïve *M. sexta* caterpillars fed diet with nicotine showed a post-ingestive feedback mechanism, indicated by reduction in biting activity after several hours of sampling the food (Glendenning, 2002). This timing suggests that the signal is communicated via a biochemical pathway initiated in the gut, rather than some chemosensory mechanism in the mouth. It is likely that the HF-fed caterpillars in our study also had a reduction in consumption rate, although we do not have data on this parameter.

In addition to the evidence that HF-fed caterpillars had decreased feeding, we also found increased excretion of fat in the fecal pellets, suggesting that there is an upper limit to fat storage above which excess must be shed. Excess dietary phosphorous in *M. sexta* larvae was mostly excreted, with little retention in the body (Woods et al., 2002), resulting in lower growth rates (Perkins et al., 2004). Our results are consistent with these findings, since caterpillars on HF diet excreted more lipid than the low fat diet caterpillars and had slower growth rates. Interestingly, we also found that despite eating less food and excreting more fat in the fecal pellets, the HF-fed caterpillars had higher lipid content in their fat bodies and fecal pellets compared to those fed on the other two diets, supporting the hypothesis that there may be an upper limit to fat storage.

Fat body is not only an organ of storage but also a site for protein and fat synthesis and intermediary metabolism (Arrese et al., 2001). Insect growth, metamorphosis and oviposition are predefined by the accumulation of nutrient reserves in the fat body (Mirth and Riddiford, 2007). Our data partially support this hypothesis because high fat diet caterpillars stored more fat in the fat body than the control group. While 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. Based solely on the amount of fat storage, one might predict that the HF diet group would have more eggs, but the decreased feeding may have caused limitations in other nutrients that were detrimental for some aspect of reproduction. It would be interesting to know what the fitness costs of the HF diet are.

High fat diet altered lipid content in the fat body and fecal pellets, suggesting that changes in fat transport may occur. We tested the hypothesis that there would be an increase in lipophorin expression. Apo I and II are proteins synthesized in the fat body to make lipophorin, (Sundermeyer et al., 1996). We used apo I and II mRNA expression as an index of lipophorin expression. In contrast to our prediction, there was no effect of diet on the expression of apo I and II in the fat body of fifth instar caterpillars. This finding is consistent with previous work showing that lipophorin synthesis did not vary with the amount of lipid in the diet (Prasad et al., 1986) and that the lipid content of lipophorin is what varies (Fernando-Warnakulasuriya et al., 1988).

Our studies have shown that early instars are not as efficient as fifth instar larvae in digesting high amount of lipid in their diet as evident by their body mass and growth rates. The midgut of fifth instar *M. sexta* shows increased rates of digestion and absorption and is therefore

able to digest ingested food better than younger larvae (Gibellato and Chamberlin, 1994). Our data on approximate digestibility of fifth shows that high fat diet caterpillars had higher approximate digestibility than the medium and low fat diet caterpillars consistent with the findings of Gibellato and Chamberlin (1994). The higher lipid content of fat body and fecal pellets can be accounted for the higher approximate digestibility of high fat diet caterpillars.

M. sexta fed high-fat diet were not obese and therefore we failed to develop a model for obesity but from our data, it appears that there may be metabolic feedback mechanisms that regulate lipid intake in insects. Most of the lipids were excreted by the HF-fed diet caterpillars, a possible mechanism for maintaining homeostasis through this feedback mechanism. The central nervous system plays an important role in nutrient regulation in *M. sexta* (Rowell and Simpson, 1992), it is likely that HF diet or the components of the lipid itself may act as stimulant signaling the brain to inhibit feeding once the threshold level of the nutrient is reached or vertebrate-like hormones may be involved in this mechanism. In drosophila melanogaster neurons in the mushroom bodies have been identified as brain centers that act as sensors of nutritional status and regulate feeding (Zhao and Campos, 2012). In mammals, short-term feeding is regulated by hormones like neuropeptide y (NPY) and ghrelin. The empty stomach acts as signal to the brain to release the hormone ghrelin, which induces feeding (Hosoda et al., 2002). NPY receptors have identified from the body and head of *Drosophila melanogaster* (Li et al., 1992) and neuropeptide F, a homologue of human NPY have also been identified in *Drosophila* (Shen and Cai, 2001). A very recent study in a gypsy moth, *Lymantria dispar* revealed the presence of hormone ghrelin in the endocrine cells of the insect midgut (Mataruga et al., 2012).

Determining the threshold level of fat storage and intake and manipulation of body fat without feeding would further validate our model of the negative feedback mechanism of

regulating body lipid content in insects. One possibility is that the effects we saw were specific to linseed oil having a high content of omega fatty acids. Therefore, using a different source of lipid and comparing the effects would also provide evidence that the lipid is acting as a signal to the brain to regulate feeding. Furthermore, studies on the effects of juvenile diet on adult morphology, flight performance, and fecundity will yield information on long-term consequences of high fat diets.

2.5. References

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