The Synthesis and Function of the Peritrophic Matrix in Drosophila Melanogaster

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THE SYNTHESIS AND FUNCTION OF THE PERITROPHIC MATRIX IN
DROSOPHILA MELANOGASTER

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ABSTRACT

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Maintenance of functional barrier structures separating an organism from and regulating its interaction with the environment is critical for many aspects of fitness. Arguably, the most dynamic interface between an animal and its environment is the gut, as the organism must balance the primary functions, digestion and nutrient acquisition, with several homeostatic and defensive systems including pathogen defense, microbiome maintenance and stem cell renewal. Many animals secrete extracellular barrier structures along their guts to organize and modulate these interactions, including intestinal mucosal secretions in humans and a semi-permeable structure in insects called the peritrophic matrix (PM). In *Drosophila*, the PM is theorized to play an integral role in numerous gut processes, but to date, all studies are limited as there are no published lines of insects that completely lack a PM.

Reported here are four genes, *drd, mco4, cyp6a22* and *cyp6t*, that produce flies completely lacking a PM when knocked down. By manipulating temporal and spatial expression of *drd*, it was found constant adult expression of *drd* is necessary for PM formation, and this phenotype is separable from other *drd* gut phenotypes. *drd, cyp6a22* and *cyp6t* are necessary for normal early
adult immune activity and defecation while mco4 is not. Global mco4, cyp6a22 and cyp6t knockdowns do not phenocopy non-gut drd mutant phenotypes.

The mco4 gene product displayed canonical laccase activity and mco4 knockdown flies were used to investigate the role of the PM in larval nutrient absorption and adult microbiome maintenance. mco4 knockdown larva develop and gain weight slower than wildtype larva and this defect is not due to food movement but rather digestive inefficiency. mco4 knockdown adult flies maintain a lower commensal bacterial load that can be abolished when stressed and conversely display a more active immune response and upregulated stem cell renewal activation.

These data support a critical role for the PM in several essential Drosophila gut functions. mco4 knockdown flies provide an ideal genetic model for studying PM function and will be a valuable tool in future studies fine tuning observations reported here and exploring additional gut processes potentially modulated by the PM.
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Chapter 1: Introduction

1.1 The Peritrophic Matrix Lines the Drosophila Gut Lumen

Every organism must interact with its environment to obtain resources while also defending itself from the multitude of threats that exist in nature. Many multicellular organisms protect themselves from and regulate their interactions with the environment through the production of cellular and extracellular epithelial barriers (Rezaee and Goras, 2014). Cellular epithelium is one of the four basic animal tissue types, along with muscle, nervous and connective tissue, and facilitate secretion, selective absorption, environmental sensing and protection (Yamaguchi et al., 2007). Extracellular epithelial barriers provide spatial segregation between cellular epithelium and the environment and from underlying tissue. Mammalian epithelial barriers interfacing with the environment can be cellular, such as the epidermis, or extracellular, such as nasal mucosal secretions, while insect environmental interfacing barriers are primarily extracellular due to the acellular exoskeleton (Kefalides et al., 2005). Maintaining integrity of epithelial barriers is essential to an organism’s survival as invasion of a pathogen, the inability to acquire an essential resource or breakdown of key homeostatic systems can lead to death (Macara et al., 2014). Numerous well studied interactions between an organism and the environment, such as digestion, oxygen acquisition and immunity, all occur at the interface of barriers separating an organism from the environment, but, while characterizing these
exchanges, there is not frequently an appreciation of how extracellular barrier structures themselves are influencing these interactions.

In animals, arguably the most dynamic interface between the organism and the environment is the gut (Gross et al., 2015). Gut epithelial barriers play essential roles in microbiome and virome regulation, digestion, pH regulation and pathogen defense (Foca et al., 2015). Even slight changes in gut homeostasis can lead to several significant health issues for an organism including malnutrition, inflammation, increased susceptibility to pathogens, diabetes, neurodegenerative diseases, and cancer (Goldwater 2015). Characterizing how barrier structures in the animal gut may influence these interactions is an understudied, but potentially very fruitful area of research.

Many species of insects produce an extracellular structure called the peritrophic matrix (PM) which lines the inside of the gut that is theorized to function analogously to human intestinal mucous secretions (Gelperin, 1971). In the model organism fruit fly Drosophila melanogaster, the PM is produced continuously throughout the fly’s life cycle in a structure anterior to the midgut called the cardia (Hegedus 2009, Figure 1.1). The PM is made of proteins embedded in a chitin polysaccharide backbone that lines the inside of the gut lumen and is semipermeable to allow the absorption of nutrients (Lehane 1997). The PM is theorized to provide a physical barrier to abrasive food particles, house a microbiome, enhance pathogen defense and aid in digestion (Hegedus 2009). However, to date, all published studies investigating PM function are limited since there are no published fly strains that completely lack a PM
Figure 1.1 Image of Adult PM

An adult fly gut was dissected and viewed under 50x magnification. The cardia and PM are labeled.

(Kuraishi et al., 2011). Nearly all studies characterizing the physiological role of the PM are based on “leaky” PM models, which allows large macromolecules to pass across the PM into the lumen of the gut that under normal conditions would be too large (Kuraishi et al., 2015). These “leaky” models can be induced either genetically by knockdown of a PM component, such as drosocrystallin (dcy) in Drosophila, or with the consumption of pharmacological agents, typically chitinase-like products in non-Drosophila studies (Kelkenboerg et al., 2014, Resch et al., 2016). Additionally, knockdown of transglutaminase, an enzyme implicated in cuticle synthesis through crosslinking of glutamine and lysine residues in multiple tissues including the PM, produces a “leaky” PM (Shibata et al., 2015). Studies using “leaky” PM models showed reduced survival, reduced
tolerance to infection and delayed development (Kuraishi et al., 2013, Kuraishi et al., 2015, Bolognesi et al., 2008, Shi et al., 2016). Ideally, an insect model that completely lacks a PM would be the best tool to characterize the physiological role of the PM.

Among insects, there is variation in gut morphology and PM secretion mechanisms (Weghofer et al., 2013). There are two classes of PM: type I which is produced typically only in the presence of food and is excreted from the midgut epithelium along the whole length of the midgut, and type II, which is secreted continuously from a structure normally at the anterior end of the midgut (Lang 2012). *Drosophila* have a type II PM as both larva and adults, however, little is known about the differences in synthesis mechanisms between the larval and adult *Drosophila* PM (Shi et al., 2004). A majority of PM research is conducted on insects, with either agricultural pest control importance, such as termites and beetles, or vector illness spreading insects, such as mosquitos, or *Lepidoptera*, and most of these insects have a type II PM as larvae and type I as adults (Liu et al., 2014). In *Drosophila*, the PM is synthesized in the cardia, a highly specialized structure with 6 separate regions of epithelial cells located just anterior of the midgut and contains at least 4 distinct layers (King 1988). The first, innermost layer of the *Drosophila* PM is very thin and originates at the cuticular surface of the stomodeal valve and is comprised of primarily of glycogen. The second layer is the thickest, containing chitin and proteins including peritrophins and chitin synthases. The third layer is comprised of small, electron-dense granules associated with epithelial cells on the outer edge of the cardia. The fourth,
The outermost layer is also very thin and comprised of electron-dense granules but is secreted by epithelial cells at the cardia-midgut junction. At least 20 genes have been identified as components of various insect PMs, with peritrophins being the most studied, and a chitin synthases have been implicated in PM production (Yin et al. 2010). *Gasp, obstructor-B (obst-B), and peritrophin-A* bind to chitin in the peritrophic matrix and are characterized as peritrophins (Behr and Hoch, 2005, Elvin et al., 1996, Shen and Jacobs-Lorena, 1997). *chitin synthase 2 (cs-2)* contributes to the synthesis of chitin in the peritrophic matrix and knockdown of cs-2 produces a “leaky” PM model (Tellam et al., 2000, Lebo et al., 2009). However, knockdown of any of these genes does not completely abolish the PM.

### 1.2 Digestive Functions and Development are Well Characterized in *Drosophila*

The primary function of the animal gut is to digest food and acquire nutrients (Sethi and Wang, 2017). Strict compartmentalization of digestive enzymes throughout the gut is necessary for maximum digestive efficiency (Li et al., 2003). The insect PM organizes the gut into three distinct regions: the endoperitrophic space surrounded by the PM, the ectoperitrophic space between the PM and the gut lumen, and PM itself (Lehane, 1997). Spatial separation of digestive properties provided by the PM confines large macromolecules such as lipids, carbohydrates and proteins to the endoperitrophic space for initial digestion (Barbehenn and Martin, 1995). The semi-permeable nature of the PM allows the passage of partially digested fatty acids, sugars and peptides from the
endoperitrophic space into the ectoperitrophic space, where classes of enzymes including carboxypeptidases, glucoamylases, trehalases, and alkaline phosphatases complete the final stages of digestion and the nutrients are eventually absorbed by enterocytes lining the lumen of the gut (Fazito do Vale et al., 2007). In the Bertha armyworm *Mamestra configurata*, there is evidence that a class of lipases are physically bound to the PM itself, underscoring the specificity of digestive enzyme location afforded by the PM (Toprak et al., 2008). Disruption of PM in the fall armyworm *Spodoptera frugiperda* eliminated the enzyme concentration gradient throughout the midgut (Bolognesi et al., 2001). It is theorized that the spatial sequestration of digestive enzymes provided by the PM is critical to digestive efficiency and studies utilizing “leaky” PM models show delayed growth rate and weight gain, presumably caused by suboptimal digestion due to the lack of organization of enzymes within the gut (Shen and Jacob 1997, Bolognesi et al., 2008).

Also enhancing digestive efficiency in numerous non-*Drosophila* insects with shorter midguts is the creation a countercurrent flow of digestive enzymes and food through the gut. Countercurrent flow is driven by water released by the posterior midgut to increase the likelihood of food and digestive enzymes interacting while traveling down the gut (Terra et al., 1988). Furthermore, the countercurrent flow of water in some insect midguts recycles digestive enzymes that travel posteriorly down the midgut back to more anterior regions, saving the animal energy by reducing the need to release new enzymes (Caldeira et al., 2007). This countercurrent flow of food and enzymes is theorized to increase
digestive efficiency and may be modulated by the organization provided by the PM. However, the extent of which and the confidence in claiming the organization provided by the PM is necessary for enzyme compartmentalization and digestive efficiency was previously limited without an insect model completely lacking a PM. Studies investigating digestive efficiency utilizing a model completely lacking a PM would provide valuable insight into the importance of the PM in a critical process.

Due to a rigid exoskeleton, insects do not grow much as adults and nearly all weight gain occurs during larval stages of development (Uryu et al., 2013). Combined with the energy requirements to undergo metamorphosis, larva typically consume food at a much more rapid rate than adult insects and therefore the most sensitive life stage to investigate digestive efficiency in insects is during larval development (Garelli et al., 2012). Drosophila larvae are a useful model for investigating digestive efficiency due to their many well characterized systems and precisely chronicled developmental timepoints. As larvae, Drosophila consume food and gain weight at a rapid rate between transitioning from an embryo until pupation (Colombani et al., 2005). Feeding and weight gain is accelerated during the final larval stage, the third larval instar (L3), when the larva is essentially obtaining vast energy stores to power through metamorphosis (Thoma et al., 2017). During L3, developmental timing and weight gain rate are incredibly sensitive to nutritional availability, and even slight changes in either food quality or digestive efficiency can significantly extend L3 and reduce the rate of weight gain (Raj et al., 2017). The Drosophila larval PM is theorized to play a
role in digestion, however this role remains undefined, but the extreme sensitivity of L3 *Drosophila* larvae to changes in digestive efficiency provide an excellent model system to study the PM role in digestion.

Another advantage for utilizing *Drosophila* L3 larvae as a model to study the role of the PM in digestion is the well-defined function of *Drosophila* insulin like peptides (dilps) in development (Wen et al., 2017). Currently, there are 8 identified *dilp* genes in *Drosophila* and each *dilp* influences organismal growth and transition to further stages of development (Sawala and Gould, 2017). Furthermore, the factors that influence expression are unique to each *dilp*, and therefore expression patterns of various *dilps* can provide insight into what is potentially causing developmental delays (Rulifson et al., 2002). For example, in larva *dilp3* and *dilp5* expression is suppressed if the organism is nutritionally stressed, while *dilp2* expression is unaffected by nutrition but altered by oxygen availability (Kannan and Fridell, 2013). Ecdysone is the primary signaling hormone to induce the transition from L3 to pupation and a spike in ecdysone expression during L3 initiates larval commitment to pupation (Handke et al., 2013). Ecdysone release or suppression is influenced by *dilp* levels in the larva and therefore *dilp* expression patterns can directly influence developmental timing (Garelli et al., 2012). *dilp8* inhibits the synthesis of ecdysone and remains at a high expression level until the larvae has acquired enough nutritional stores to transition to metamorphosis, when its expression then drops dramatically (Garelli et al., 2012, Uryu et al., 2013). Hence, a profile of *dilp* expression in larva can display how the organism is being stressed and provide researchers
more confidence in pinpointing the cause of any developmental defect. This sensitivity potentially allows very in-depth examination of when and the extent of which the larval PM may affect nutritional uptake.

1.3 *Drosophila* as Model to Study Innate Immunity

All animals have some form of innate immunity to protect themselves from the environment, and the *Drosophila* gut has served as a model to study innate immune responses for decades (Lemaitre and Hoffman 2007). Unlike mammals, *Drosophila* do not have an adaptive immune system, so they rely completely on their innate system for organismal defense (Buchon et al., 2014). While feeding, the animal gut is susceptible to ingestion of pathogenic bacteria (Loch et al., 2017). The *Drosophila* gut has four basic levels of defense from pathogens: physical protection afforded by the PM, release of antimicrobial peptides into the lumen by the *immune deficient* (*imd*) and *toll* pathways, release of ROS into the gut lumen primarily by *Dual oxidase* (*Duox*), and rapid regeneration of damaged epithelial cells by the stem cell *JAK/STAT* pathway (Hori 2017). "Leaky" PM flies die more rapidly when exposed to pathogens (Kuraishi et al., 2013, Shibata et al., 2015) and mutation to components of the *imd*, *toll* and *JAK/STAT* pathways also leads to reduced survival upon pathogen ingestion (Tepas et al., 2001, Huszar and Imler, 2008, Wang et al., 2010, Shokal et al., 2016). Bacterial invasion leads to an upregulation of the *imd* and *toll* pathways, which can be quantified by the expression of the antimicrobial peptides produced by each pathway, *dipterinc* (*Dpt*) and *Drosomyocin* (*Drs*) respectively (Lee and Lee
Gram negative bacteria typically induce upregulation of the *imd* pathway while gram positive bacteria generally induce upregulation of the *toll* pathway, however there is a slight upregulation of both pathways in the presence of either type of bacteria (Buchon et al., 2014). Bacteria are recognized by the fly based on the peptidoglycan (PGN) structure found on most bacteria by PGN-recognition proteins (PGRPs), of which *Drosophila* have 13 genes encoding 19 different proteins (Werner et al., 2000). PGRPs induce signaling cascades that activate the *toll* and *imd* pathways with PGRP-SA and PGRP-SD inducing the *toll* pathway and the remaining *Drosophila* PGRPs either positively or negatively regulating the *imd* pathway (Werner et al., 2003). In the *toll* pathway, the binding of bacterial PGNs to receptors activates *spätzle* (*spz*), which induces a conformational change in TOLL (Gottar et al., 2002). Activated TOLL leads to the induction of a series of intracellular signaling cascades that ultimately leads to the nuclear translocation of nuclear factor-kappa B (NF-κB) proteins DIF and DORSAL, which induces transcription of the antimicrobial peptide *drs* (Lindsay and Wasserman 2014). In the *imd* pathway, the binding of bacterial PGNs to receptors recruits a signaling complex including *imd, dredd* and *dfadd* (Lemaitre et al., 1995). DREDD cleaves a 30 amino acid sequence of IMD which allows IMD to be ubiquinated by LAP2 (Hanson et al., 2016). The ubiquinated IMD activates the *Drosophila* IKK complex, which phosphorylates the transcription factor *Relish* (Buchon et al., 2014). Phosphorylated RELISH induces transcription of *dipterincin* and additional antimicrobial peptides (Mistry and Kounatidis, 2014). Release of *Drs* and *Dpt* into the gut lumen reduces gut bacterial load, and
measuring the levels of Drs and Dpt expression is a reliable mean for researchers to quantify immune system induction (Buchon et al., 2015).

In addition to the Toll and imd pathways, Duox mediated ROS production in the gut acts as a form of immunity and modulates bacterial populations in the gut (Buchon et al., 2015). Duox is a NADH oxidase integral membrane protein localized to the cell surface and releases ROS into the gut lumen in the presence of bacteria (Ha et al., 2005). Reduction of Duox leads to dysbiosis of gut cell proliferation and an increase in gut bacterial load (Ha 2009). Knockdown of Duox results in changes in levels of Saccharomyces cerevisiae, a primary food source, throughout the gut (Ha et al., 2005). mesh regulates Duox expression and knockdown of mesh leads to an increase in gut bacterial load (Xiao et al., 2017). Knockdown of Duox also increases expression of Drs and Dpt, suggesting the toll and imd pathways can at least partially compensate for loss of Duox function for combating microbial invasion in the gut (Ha 2009). The toll and imd pathways, and Duox all contribute to gut innate immunity and bacterial load in the gut, and the activation of these pathways is quantifiable by researchers, providing a model to study host-bacterial interactions.

The JAK/STAT stem cell renewal pathway is canonically induced upon cell wounding (Lee et al., 2017). The JAK/STAT pathway is well conserved from insects to mammals and stands for JAnus Kinase protein and the Signal Transducer and Activator of Transcription (Leonard 2001). Upon cellular damage, UPD3 is released into the hemolymph of adult flies and binds to its original and nearby cells membrane-bound DOMELESS (DOME) receptors
(Brown et al., 2001). This binding activates the JAK tyrosine kinase *hopscotch* (*hop*) to phosphorylate itself and the cytoplasmic tail of DOME, which creates docking sites for STAT family of proteins (Binari 1995). STAT proteins, primarily STAT92E in *Drosophila*, are phosphorylated by HOP and translocate into the nucleus, where they induce transcription of genes implicated wound healing and cell proliferation (Yan et al., 1996). Researchers often quantify the activity of the JAK/STAT pathway, and indirectly damage response and wound healing, by measuring the level of *upd3* expression (Zhang et al., 2017). The extent to which the PM protects the *Drosophila* gut from bacterial infection remains unknown, but the deep understanding of immune response and wound healing pathways in *Drosophila* allow for precise read outs of overcompensation and damage response in a model lacking a PM.

**1.4 *Drosophila* is Leading Model to Study Host-Microbiome Interactions**

In part due to the expansive knowledge of their immune and homeostatic systems, *Drosophila* has emerged as a leading model to study host-microbiome interactions in recent years (Sleiman et al., 2015). Commensal bacterial populations play integral roles in digestion, pathogen defense and stem cell turnover (Broderick et al., 2012, Fink et al., 2013, Bost et al., 2017). Ablation or augmentation of commensal microbial diversity can lead to changes in host transcriptional regulation, life span, reproductive success and metabolism (Lizé et al., 2014, Yuval 2017). The typical laboratory raised *Drosophila* gut microbiome contains 6-12 families of bacteria with *Acetobacter* (60-80%) and
Lactobacillus (10-20%) dominating the total abundance (Early et al., 2017).

Human gut microbiomes can contain over 1000 different families of bacteria per individual (Ewald and Sumner, 2018). Additionally, the Drosophila laboratory raised microbiome is very easily manipulated experimentally, as treatment with antibiotics can nearly completely remove a commensal bacteria population and reintroduction of one or a few members of the microbiome can lead to those members comprehensively occupying the entire microbiome niche of the fly's gut (Thibert et al., 2016). Transfer of flies to fresh vials daily can stress the microbiome and greatly reduce the total gut bacterial load, allowing for easy experimentation to test the resiliency of the microbiome (Blum et al., 2013).

Drosophila and their gut microbiome are intricately associated with one another (Broderick et al., 2014). The microbiome is necessary for optimal digestive efficiency and ablation of the microbiome results in smaller flies and impaired insulin signaling (Erkosar et al., 2017). Upregulation of the toll and imd pathways reduce the gut bacterial load (Schroeder et al., 2008). The commensal bacteria population release a higher level of uracil than non-native bacteria, and the release of uracil reduces activation of the toll and imd pathways (Kim et al., 2014). Two high population members of the microbiome, Acetobacter pomorum, and Lactobacillus plantarum, are necessary for optimal insulin and TOR signaling efficiency in adult flies (Shin et al., 2011). Maintaining a stable microbiome is necessary for the health of the fly as it has been shown that a member of the commensal microbiome, Gluconobacter morbifer, can become pathogenic if its population becomes too large (Buchon et al., 2009). The commensal
microbiome also enhances *Drosophila* immunity as ablation of the microbiome increases the susceptibility of infection (Zhai et al., 2017).

The simplicity and the technical ease of augmentation of the microbiome coupled with the knowledge and power of *Drosophila* genetic tools enlist *Drosophila* as an attractive model for investigating host-microbiome interactions. Despite *Drosophila* only recently being adopted as a major model for host-microbiome relationships, several meaningful observations using *Drosophila* have been made about the role of the microbiome on host digestion, metabolism, immunity, reproductive success and survival (Venu et al., 2014, Flaven-Pouchon et al., 2014, Newell and Douglas, 2014, Mast et al., 2014, Ye et al., 2016). Even with a boom of interest in *Drosophila* microbiome research, there currently are no published studies investigating the role of the PM in modulating host-microbiome relationships. The PM could potentially be a major factor in establishing and maintaining a microbiome due to its theorized importance in gut compartmentalization, and investigation of how the PM may influence a number of host-microbiome relationships could give insight into PM function and serve as an influential model of gut barrier-microbiome interactions.

### 1.5 drop-dead Mutant Flies Lack a PM

Perhaps because no genetic model completely abolishing the PM existed previously, there has been little work done investigating the physiological role of the PM, and additionally, most PM physiological studies were conducted in insects with agricultural or vector illness importance (Hegedus 2009). However,
the genetic tools available in *Drosophila* along with the many well characterized systems can allow for more in-depth analysis of PM physiology in *Drosophila* compared to other insects. For decades, *Drosophila* has been used to study digestion and innate immunity, and recently, *Drosophila* has emerged as a leading model to study host-microbiome relationships (Nehme et al., 2007, Gulden et al., 2015, Foca et al., 2015). How the PM may be influencing these well-defined gut dynamics is largely unknown but could potentially be easier to answer experimentally in *Drosophila* compared to other insect models.

Our lab has recently observed mutation to the *Drosophila* gene *drop-dead* (*drd*) produces adult flies that completely lack a PM (Conway et al., 2018). Mutation of the gene *drop-dead* (*drd*) leads to multiple adult phenotypes including female sterility, neurodegeneration, early adult lethality and defective gut function (Blumenthal, 2008, Peller et al., 2009). The *drd* gene encodes a member of the NRF-domain family of proteins which have limited homology to a family of bacterial acyltransferases however the biochemical function of DRD has not yet been determined (Blumenthal 2008). In *drd* mutant adult flies, food movement through the digestive system is inhibited, as indicated by reduced defecation rates and slower movement of dyed food through the gut (Blumenthal 2008, Peller et al., 2009). Additional *drd* mutant gut phenotypes include an enlarged crop and depletion of triglyceride and glycogen stores. Therefore, it appears that *drd* mutant flies exhibit a starvation phenotype despite ingesting food (Peller et al., 2009).
*drd* mutants display early adult lethality, however, it has been shown that expression of *drd* in the trachea during metamorphosis is sufficient to rescue early lethality (Sansone and Blumenthal 2012). Additionally, knockdown of *drd* during adulthood does not affect survival, suggesting *drd* plays a critical developmental role during metamorphosis. While *drd* mutants lack a PM, it remains unknown what the spatial and temporal patterns of *drd* expression is necessary for PM formation, and if lacking a PM contributes to the early adult lethality of *drd* mutants.

While *drd* mutants exhibit many phenotypes, often they can be separated. It was previously hypothesized that the defect in food movement through the gut in *drd* mutants might be a secondary consequence of neurodegeneration and the resulting loss of neuronal control over the stomodaeal valve in the cardia (Peller et al., 2009). However, in later work, the two phenotypes were separated by manipulating *drd* expression specifically in the respiratory tracheae (Sansone and Blumenthal, 2013). When *drd* expression is knocked down in the tracheal system, flies exhibit neurodegeneration and early adult lethality (median lifespan of 5 days), but not gut dysfunction, as measured by defecation rate and nutrient storage. Additionally, rescue of *drd* expression in the tracheae in a *drd* mutant background rescues neurodegeneration and slightly extends adult lifespan (median lifespan of 8 days) without rescuing defecation rate. Therefore, the neurodegenerative and gut dysfunction phenotypes are independent, with the first associated with *drd* expression in the tracheae and the tissue dependence of the second still undetermined. In addition, the extent to which *drd* mutant gut
dysfunction contributes to early adult lethality remains unknown. While the pleiotropic nature of \textit{drd} mutants could render potential experimental results difficult to interpret, the ability to separate complicating phenotypes allow the use of \textit{drd} mutants to serve as an improved model for studying PM synthesis and function in \textit{Drosophila} compared to “leaky” PM models. Ideally, a genetic model lacking a PM with less pleotropic effects could better characterize PM function and synthesis.

\textit{drd} mutants display diverse phenotypes across a range of tissues, but it remains unknown what genetic and biochemical pathways \textit{drd} may interact with and if \textit{drd} is interacting with the same factors in different tissues (Blumenthal unpublished). Unpublished data from our lab and a study in worms suggest \textit{drd} may be involved in lipid processing. One of the phenotypes of \textit{drd} mutants is early adult lethality. In an enhancer screen, \textit{oysgedart (oys)} and \textit{spinster (spin)} showed an increase in lethality for \textit{drd} mutants (Blumenthal unpublished). Both \textit{oys} and \textit{spin} are lipid processing genes, implying \textit{drd} may be involved in similar pathways. \textit{oys} is a membrane-bound O-acyltransferase that adds acyl tails to unsaturated fatty acids and its expression in the germline is necessary for normal \textit{Drosophila} germline development (Steinhauer et al., 2009). \textit{spin} is predicted to have sphingolipid transporter activity which facilitates the movement of sphingolipids, lipids containing the long-chain amine diol sphingosine or a closely related base, either within or between cells, and is necessary for normal adult \textit{Drosophila} eye development (Yuva-Aydemir et al., 2011). Studies using the \textit{C. elegans} homologues of \textit{drd}, \textit{ndg-4} and \textit{nrf-6}, suggest a role in lipid processing
(Watts et al., 2006). When worms are fed DGLA, a long chain omega-6 polyunsaturated fatty acid, wildtype worms are sterile due to degradation of germ cells, suggesting DGLA promotes increased apoptosis in germ cells. When *ndg*-4 and *nrf*-6 were mutated, the worms were fertile. This result implies *drd* homologues in worms are lipid transporters and play a role in lipid processing since there is no apoptotic signal derived from the fatty acid. However, targets of this activity and other genes involved in potential *drd* lipid processing pathways remain unknown.

Microarray data from stage 10 egg chambers in *drd* knockdowns showed a significant change in expression of three families of genes compared to wildtype: cytochrome p450s, juvenile hormone epoxide hydrolases (JHEH), and glutathione S transferase epsilons (Blumenthal unpublished). The change in expression of these three families in *drd* knockdowns may be evidence they are functioning in similar pathways. Cytochrome p450s are hemoproteins, meaning they contain a ferrous binding site and typically require an iron cofactor for redox activity (Estabrook et al., 2003). The *Drosophila melanogaster* genome contains 90 cytochrome p450 genes, most of which have undetermined function (Good et al., 2014). All studied cytochrome p450 genes whose function has been analyzed show the ability to metabolize either exogenous or endogenous substrates and have therefore been linked to hormone and signaling pathways in addition to their most commonly studied function: insecticide resistance (Bass et al., 2015). A large portion of cytochrome p450 family members are found in the midgut, where they metabolize potentially harmful chemicals into ones that will
not damage the fly, hence why they are studied in the context of insecticide resistance. JHEH add water to reactive epoxides and are often associated with increased tolerance to oxidative stress (Jia et al., 2017). Glutathione S transferase epsilons are metabolic isozymes shown to catalyze the reduction of glutathione (Udomsinprasert et al., 2005). Typically, Glutathione S transferase epsilons are often associated with detoxification of xenobiotic substrates by facilitating a nucleophilic attack of non-polar sidechains of foreign molecules to prevent their interaction with critical cellular proteins and substrates (Tinta et al., 2012). It remains unknown if *drd* is interacting cytochrome p450s, JHEHs, and glutathione S transferase epsilons, or if any genes from these families are also necessary for PM synthesis, but are intriguing candidates to investigate.

The following work investigates the genetic requirements for *Drosophila* PM formation and the physiological role of the PM in several gut and whole organismal systems. Observations made here present powerful models for studying the PM and provide novel insight into *Drosophila* PM synthesis and function.
Chapter 2: Materials and Methods

*Drosophila* stocks and maintenance

All fly stocks were maintained on standard cornmeal-yeast-agar food (http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/molassesfood.htm) at 25°C on a 12h:12h light: dark cycle. For heat-shock experiments, flies were kept at 30°C for the time periods described in the results. For RNAi experiments, a *UAS-Dcr-2* transgene was included in the genetic background of the flies in order to boost RNAi efficiency; the *drd*<sup>GD15915</sup> *UAS-Dcr-2* and *UAS-Dcr-2* *drd*<sup>GD3367</sup> lines were created previously by recombination between VDRC stocks *w<sup>1118</sup>;P{GD3367}v37404* (FBst0461992) and *w<sup>1118</sup>;P{GD15915}v51184* (FBst0469325) and Bloomington stock *w<sup>1118</sup>;P{UAS-Dcr-2.D}2* (FBst0024650, RRID:BDSC_24650) (Sansone and Blumenthal, 2013). Other stocks (*w<sup>1118</sup>;P{UAS-GFP.nls}14* (FBst0004775, RRID:BDSC_4775), *w<sup>*</sup>;P{GAL4-Hsp70.PB}2* (FBst0002077, RRID:BDSC_2077), *w<sup>+</sup>;P{UAS-lacZ.B}Bg4-2-4b* (FBst0001777, RRID:BDSC_1777), *w<sup>1118</sup>;P{GawB}DJ717* (FBst0008180, RRID:BDSC_8180), *w<sup>1118</sup>;P{GawB}DJ626* (FBst0008166, RRID:BDSC_8166), *w<sup>1118</sup>;P{GawB}DJ628* (FBst0008167, RRID:BDSC_8167), *y<sup>1</sup> w<sup>*</sup> Mi{y<sup>+</sup>mDint2=MIC}drd<sup>M15121</sup>/FM7h* (FBst0059743, RRID:BDSC_59743), *y<sup>1</sup> w<sup>*</sup>;P{w<sup>+</sup>mC=loxP(Trojan-GAL4.1)}12B; Dr<sup>1</sup>/TM3, Sb<sup>1</sup>* (FBst0060304, RRID:BDSC_60304), *y<sup>1</sup> w<sup>67c23</sup>; sn<sup>a</sup>Sco/CyO, P{w<sup>+</sup>mC=Crew}DH1* (FBst0001092, RRID:BDSC_1092), *y<sup>1</sup> M{3xP3-RFP.attP}ZH-2A w<sup>+</sup>; M{vas-int.Dm}ZH-102D* (FBst0024480, RRID:BDSC_24480), and *FM7i/C(1)DX, y<sup>1</sup> f<sup>1</sup>* (FBst0005263, RRID:BDSC_5263)) were obtained from the
Bloomington *Drosophila* Stock Center. The line bearing the second chromosome
*UAS-dr* on a *drd<sup>lwf</sup>* background was described previously [Sansone and
Blumenthal, 2013]. The *drd* genes and alleles referenced in this work include *drd*
(FBgn0260006), *drd<sup>lwf</sup>* (FBal0193421). Lines used in genetic screens are listed
in chapter 4 and were acquired from the Vienna and Bloomington Stock Centers.
*DA-gal4* and *IMD<sup>1</sup>* stocks were acquired from Bloomington Stock Center. Stocks
were not outcrossed prior to this study.

For rescue experiments with two copies of the *DJ717-gal4* driver, the
*UAS-dr* transgene was recombined onto the *DJ717-gal4* driver chromosome by
a standard crossing scheme. Recombinants were identified by PCR (GoTaq Hot
Start Polymerase, Promega, Madison, WI). Primers used to detect the *UAS-dr*
transgene were pUAST 3’ seq: 5’ CAG TTC CAT AGG TTG GAA TC 3’ and
CG5652 6a: 5’ GAT CGC CTG GTG TTT GTT TT 3’. The resulting recombinant
chromosome was crossed onto a *drd<sup>lwf</sup>* background. Additional lines used in this
work include *imd<sup>1</sup>* (FBst0055711) and *da-gal4* (FBtp0001168).

**Calcofluor Staining**

Calcofluor (Sigma-Aldrich, St. Louis, MO) was prepared in water as a 1%
solution. For staining procedure 1, two-day old flies were fed with a 1:3 mixture
of calcofluor solution and 1% sucrose for 6 hr-overnight. Midguts were then
dissected in PBS, fixed in 4% PFA/PBS for 30 min, washed 3x10 min in PBS and
mounted on slides. For staining procedure 2, midguts of two-day old flies were
dissected and placed into a 1% calcofluor solution for 30 minutes. The guts were
then fixed, washed, and mounted as above. Samples were imaged on a Nikon
A1 Confocal Microscope (Nikon, Tokyo, Japan) with NIS-Elements AR software (Nikon).

**Lifespan Assays**

Flies were collected on the day of eclosion, transferred to fresh vials every 2-7 days, and scored daily for survival for 40 days. A minimum of 50 flies per genotype were used for each survival curve.

**Defecation Assays**

Assays were performed as previously described (Blumenthal 2008). Briefly, two male flies were placed in a vial containing instant food (Carolina Biological, Burlington, NC) prepared with 0.5% Acid Blue 9 on the day of eclosion (unless otherwise indicated in the text). After 24 hours, the flies were transferred to a fresh vial. After another 24 hours, the blue fecal spots on the vial were counted.

**PM Screen**

2-3 day old offspring of *DJ626* and various UAS-RNAi lines were collected and scored for the presence of a PM based on gross dissection. Fly guts were visualized under a dissecting scope and scored for the presence of a PM based on appearance or absence of the PM in the midgut. At least 10 flies per genotype were assayed.
Quantitative Real Time PCR

Larvae at various timepoints in L3 were collected and homogenized. RNA was isolated and DNase treated (RNaqueous Micro kit, Thermo Fisher Scientific, Waltham, MA), and 50% of each RNA prep was then used as a template for cDNA synthesis (Qscript, Quansys Biosciences, Logan, UT). Quantitative PCR was performed using Perfecta SYBR mastermix (Quansys), 250 nM primers, 0.1 μL cDNA per reaction in a CFX thermocycler (Bio Rad, Hercules, CA) and analyzed using CFX Manager software (Bio Rad). Each PCR run contained dilution series of one cDNA with all tested primer sets, and the resulting standard curves were fit with straight lines ($r^2 \geq 0.98$, efficiency 85–101%) and used to quantify unknowns. Levels of various dilp expression was normalized with the housekeeping gene rp49.

RT Primers Used

rp49 Forward: AAGATCGTGAAGAAGCGCACCAA
rp49 Reverse: CTGTTGTCGATACCCTTGGGCTT
dilp2 Forward: CGAGGTGCTGAGTATGGTGTG
dilp2 Reverse: CCCCAAGATAGCTCCCAGGA
dilp3 Forward: GTGTATGGCTTCAACGCAATG
dilp3 Reverse: CAGCAGGAACGGTCTTC
dilp5 Forward: TGCCTGTCCCAATGGATTCAA
dilp5 Reverse: GCCAAGTGGTCCTCATAATCG
dlp8 Forward: CCTGGCTGCACGTGAACTAT
dlp8 Reverse: TGGATGGACAATACTCGGCG
16S-341F: CCTACGGGAGGCAGCAG
16S-907R: CCGTCAATTCTTTTRAGTTT
diptericin R: TGGTGGAGTGGGCTTCATG
diptericin F: GCTGCGCAATCGCTTCTACT
upd3 R: CCAGTGCAACTTGATGTTGC
upd3 F: GAGCACCAAGACTCTGGACA
drosomycin R: TCCCAGGACCACCAGCAT
drosomycin F: CGTGAGAACCCTTTTCCAATATGATG

**UAS and GAL4 Plasmid Construction**

UAS and GAL4 plasmids were constructed using a series of Addgene vectors: 17574, 17575, 26226, 26227. UAS and GAL4 sequences were inserted using Promega Gateway cloning methods. The mco4 transcript sequence was amplified from DGRC clone FMO06372, which contained the mco4 ORF, using primers at the start and end of the mco4 transcript sequence: ACTGACAGTAACATTTGGCCC and GTTAACTTTTAGCTGG. The mco4 GAL4 sequence was determined as the 844 bp region between mco4 and the neighboring gene and was amplified from genomic DNA using primers at the beginning and end of the sequence: CACACAGATCAGCCGACTGC and CCGAGCATTGAACAAATGTG.

**MCO4 CRISPR Plasmid Construction**

20 bp guide RNA sequences were spliced into Addgene plasmid #49408, pCFD1-dU6:1gRNA, via restriction enzyme cloning, and sent to BestGene for injection. The guide RNA sequences targeted the third and fourth exon with
sequences: TTCGATGTATCCGCAGCTCTCCTC and TTCGTTATCGATGCTG.
AAGCTGAT. Detection of mutants was determined by PCR of an 800 bp region around the guide RNA site and a cel-1 assay from female flies that could potentially be heterozygous for a mutation (cel-1 provided by Petrella lab). Cel-1 enzyme mix was diluted 20-fold with water and mixed at a 3:2 ratio of PCR product and heated to 42\(^o\) for 20 minutes. The Cel-1 reaction product was run on a gel and a potential mutant was identified if there was a band around 400bp in length.

**Larval Collections**

Larvae were collected on 35mm dishes containing laboratory fly food.

**Larval Timing**

Larvae were raised in 35 mm dishes with laboratory fly food and scored as entering L3 upon branching of the anterior spiracles and darkening of the posterior spiracles. Eggs were collected on 35mm dishes and designated to 1-hour time cohorts based on transition from embryos to L1, and allowed to grow into L3 larvae. Upon entering L3, larvae were transferred to vials and monitored hourly until pupation.

**Body Mass Measurements**

Two-day old adult flies were frozen and killed with dry ice, allowed to return to room temperature and massed individually.
Larval Fecal Quantity Assay

Upon reaching 24 hours into L3, larvae raised on normal laboratory food were placed on normal laboratory food dyed with 1% acid blue at a ratio of 50ml of food to 1ml of 1% acid blue dye for one hour. Individual larva were then transferred to 0.75ml microcentrifuge tubes filled with 0.2ml of apple juice agar (recipe for 100ml: 75ml water, 25ml apple juice, 2.5g sucrose, 2.225g *Drosophila* agar, 0.15g tagosept) for 2 hours. The larvae were then removed and 0.2ml of 1xPBS was added to each microcentrifuge tube. The tubes were raised to 70°C until the agar melted and 5 tubes with the agar/PBS mixture were combined. The absorbance of each sample maintained at 70°C was measured at 625nm on a spectrophotometer.

Larval Feeding Quantification

Upon reaching 24 hours into L3, larvae raised on normal laboratory food were placed on normal laboratory food dyed with 1% acid blue at a ratio of 50ml of food to 1ml of 1% acid blue for one hour. Five larva per genotype were then added to a microcentrifuge tube with 1ml of PBS and homogenized. The absorbance of each sample was measured at 625nm on a spectrophotometer.

Larval Gut Contraction Quantification

Larvae 24 hours into L3 were stuck to a microscope slide with double-sided tape dorsal side down. Under the view of a dissecting scope, the number of gut contractions in three distinct regions: the anterior midgut, the acidic midgut
and the posterior midgut, were counted for 3 two-minute increments per larva, per region. The average of the 3 scores per larva per region were reported.

**Nutri-Fly™ “German Food” Sick Fly Formulation**

One liter of Nutri-Fly™ “German Food” Sick Fly Formulation was prepared by mixing 1 liter of water with a pre-prepared package of formulation sent from FlyStuff and brought to a boil. After ten minutes of boiling the mixture was lowered to 60°C and 1.4 g of tagosept dissolved in 14mL of 95% ethanol was added. Food was dispensed into vials and cooled overnight.

**pH Staining**

Flies were fed a mix of 1% sucrose with 1% Sigma m-Cresol purple pH dye in water for 5 hours. Guts were dissected and visualized under a dissecting scope.

**Bacterial DNA Extraction**

Bacteria was extracted from whole flies that were washed with water and ethanol then using MO Bio PowerSoil DNA Isolation Kit. Each extraction was from at least 10 flies.

**Bacterial Sequencing**

Microbial community composition was determined by 16s Ribosomal PGM sequencing performed by Molecular Research Laboratory, 503 Clovis Rd
Shallowater, TX 79363. Sequencing was 20k reads of 300bp with 515F 16S primers.

**Antibiotic Food Preparation**

Normal laboratory food was heated and melted. Carbenicillin (50ug/ml) and chloramphenicol (50ug/ml) were added and the new food was poured into vials.
Chapter 3: Continuous Expression of *drop-dead* in the Adult Cardia is Necessary for PM Synthesis

3.1 Introduction

Obtaining energy from food is vital for animal survival, and deviations from normal digestive function can have enormous impacts on organismal fitness (Aliyar et al., 2015). The gut of *Drosophila melanogaster* is a well characterized model for studying digestive function (Guo et al., 2016). In wildtype adult flies, food is stored in an organ called the crop. For digestion to occur, the crop contracts and food passes through the stomodaeal valve of the cardia into the midgut (Geleprin et al., 1971; Stoffolano 1995). As in many other insect species, the midgut of both larval and adult *Drosophila* is lined with a semi permeable extracellular epithelial barrier called the peritrophic matrix (PM) that is theorized to aid in digestion and to protect the gut from pathogens and physical abrasion (Hegedus 2009). Nutrients pass through the PM and are absorbed by the gut epithelial enterocytes (Lemaitre 2013).

Mutation of the *Drosophila* gene *drop-dead* (*drd*) leads to multiple adult phenotypes including female sterility, neurodegeneration, early adult lethality and defective gut function (Blumenthal, 2008; Peller et al., 2009). The *drd* gene encodes a member of the NRF-domain family of proteins which have limited homology to a family of bacterial acyltransferases and for which a biochemical function has not yet been determined (Blumenthal 2008). In *drd* mutant flies, the
crop becomes enlarged, and food movement through the digestive system is inhibited, as indicated by reduced defecation rates (Blumenthal, 2008, Peller et al., 2009). Additionally, triglyceride and glycogen stores are reduced in the mutants. Therefore, it appeared that *drd* mutant flies display a starvation phenotype despite ingesting food, however, the cause of these gut dysfunction phenotypes remains unknown (Peller et al., 2009).

Since *drd* mutants display a range of phenotypes across various tissues, an ongoing major goal of our lab is to separate these phenotypes and determine the causal relationships between them. It was previously hypothesized that gut dysfunction in *drd* mutants might be a caused by neurodegeneration and the resulting loss of neuronal control over the stomodaeal valve in the cardia (Peller et al., 2009). However, these two phenotypes were separated by manipulating *drd* expression specifically in the tracheae. When *drd* expression was knocked down in the tracheal system, flies exhibited neurodegeneration and early adult lethality (median lifespan of 5 days), but not gut dysfunction, as measured by defecation rate and nutrient storage. Additionally, rescue of *drd* expression in the tracheae in a *drd* mutant background rescued neurodegeneration and extended adult survival (median lifespan of 8 days) without rescuing defecation rate. Therefore, the neurodegenerative and gut dysfunction phenotypes are independent, with the first associated with *drd* expression in the tracheae and the cause of the second still undetermined. In addition, the extent to which *drd* mutant gut dysfunction contributes to early adult lethality remains unknown.
In this chapter, I report a novel and unique gut phenotype of *drd* mutants: lack of a PM. This is a significant observation as to date there are no published fly lines that completely lack a PM (Kelkenberg et al., 2015). Additionally, I explored the spatial and temporal expression requirements of *drd* for PM synthesis, investigated the physiological consequences of lacking a PM, and attempted to uncouple the lack of PM phenotype and additional *drd* mutant phenotypes. I find that continuous expression of *drd* in the cardia is necessary for PM formation, but surprisingly, lacking a PM is not the sole cause of *drd* mutant gut dysfunction nor does lacking a PM appear to contribute to early adult lethality.

### 3.2 *drd* Mutants Lack a PM as Adults but Have a PM as Larvae

Our lab has previously reported defective gut function in *drd* mutant adults, as indicated by enlarged crop volumes, slow movement of food into the midgut, and decreased defecation (Blumenthal 2008, Peller et al., 2009). I now report a structural abnormality in the guts of adult *drd* mutants, namely the absence of the PM. The PM can be visualized by gross dissection of the midgut as a transparent, tough membrane running down the lumen of the midgut. A PM was always observed in more than 50 dissections of wild-type flies or females heterozygous for the severe alleles *drd* or *drdlwf*, but we never observed a PM in at least 50 dissections of *drd* or *drdlwf* hemizygous males or homozygous females (from 2-day to a week old). To confirm the absence of the PM in *drd* mutant adults, we imaged dissected midguts of *drd* mutants after feeding with the
chitin-binding dye calcofluor. Wildtype midguts (two day old \textit{drd}^{lw}/fm7a females) show a bright, clear, structured staining down the midgut, indicating the presence of a PM, while \textit{drd} mutant midguts (two day old \textit{drd}^{lw}/\textit{drd}^{lw} and \textit{drd}^{1}/\textit{drd}^{1} females) show a weak, diffuse staining, indicating the absence of a PM (n=4 \textit{drd}^{lw}/fm7a, n=4 \textit{drd}^{1}/fm7c, n=4 \textit{drd}^{lw}/\textit{drd}^{lw} and n=2 \textit{drd}^{D1}/\textit{drd}^{D1}, data not shown). Because \textit{drd} mutant flies have a defect in the movement of food into their midgut, the absence of calcofluor staining in mutants could result from a failure of the dye to reach the PM. To control for this possibility, dissected midguts from wild-type and \textit{drd} mutant flies were incubated in calcofluor, and again, a clearly stained PM was observed in the wild-type but not the mutant midguts (Figure 3.1).

To determine if \textit{drd} mutant larva also lacked a PM, I dissected male \textit{drd} mutant larval guts investigating for the presence of a PM. Interestingly, 16/16 \textit{drd}^{lw} hemizygous male larvae had a PM. These results indicate that while \textit{drd} is necessary for PM formation in adult flies, \textit{drd} is not necessary for PM formation in larvae.

### 3.3 Expression of \textit{drd} in the Cardia is Necessary for PM Synthesis

Because the PM is synthesized in the cardia, I examined the effects of manipulating \textit{drd} expression with three gal4 drivers with differing levels of expression in the cardia then investigating for the presence of the PM. The \textit{DJ626} driver showed robust expression in the anterior cardia of both late pupae and young adults, the \textit{DJ717} driver showed mild expression only in the adult, and the \textit{DJ628} driver was not expressed in the cardia (Figure 3.2, Sansone
Figure 3.1 *drd* Mutants Lack PM as Adults

Two-day old female fly guts were stained with the chitin binding fluorophore calcofluor to assay for the presence of a PM. (A) *drd^{wr}/fm7a* guts show a clear, structured staining, indicating the presence of a PM, while (B) *drd^{wr}/drd^{wr}* guts display a weak, diffuse staining, indicating the absence of a PM.

Figure 3.2 Driver Lines Display Varied Levels of Expression in Cardia

Three GAL4 driver lines were examined for their level of expression in the cardia. The *UAS-LacZ* reporter line was crossed with the *DJ626-Gal4* (A), *DJ717-Gal4* (B), and *DJ628-Gal4* (C) driver and x-gal staining was performed on the digestive system of 2-day-old flies. The full digestive system was imaged at 5x and the cardia was imaged at 20x. From Conway et al., 2018 and Sansone dissertation 2013.
dissertation 2013, Conway et al. 2018). Adult flies with \textit{drd} knocked down by DJ626 and DJ717 lacked a PM, while flies with \textit{drd} knocked down by DJ628 still had a PM as adults (Table 3.1). \textit{drd} expression was rescued by the three driver lines in a \textit{drd} mutant background and only a rescue of \textit{drd} in the DJ626 pattern restored the PM. These results indicate strong expression of \textit{drd} in the cardia is necessary for PM formation in adult flies.

3.4 Expression of \textit{drd} during Adulthood is Necessary and Sufficient for Adult PM Synthesis

In \textit{Drosophila}, the PM is synthesized continuously throughout life (Hegedus 2009). To determine the temporal expression requirement of \textit{drd} for PM synthesis, I utilized a heat shock inducible promoter to knock down or rescue \textit{drd} at various points in development, then assayed for the presence of a PM. It has previously been reported that \textit{drd} expression is only necessary during metamorphosis for adult survival (Sansone and Blumenthal, 2013), therefore, it is possible to knockdown \textit{drd} during adulthood or rescue \textit{drd} in a mutant background during metamorphosis without inducing early adult lethality exhibited by \textit{drd} mutants. I performed three \textit{drd} developmental knockdown experiments to elucidate the temporal expression pattern of \textit{drd} necessary for PM formation in adults: 1) knocking down \textit{drd} on the first day of eclosion for 7 days then assaying for the PM, 2) knocking down \textit{drd} only during metamorphosis then assaying for the presence of a PM 3 days post eclosion, 3) rescuing \textit{drd} in a \textit{drd} mutant
Table 3.1 Robust Expression of *drd* in the Cardia is Necessary for PM Formation

<table>
<thead>
<tr>
<th></th>
<th>Strong DJ626</th>
<th>Mild DJ717</th>
<th>Minimal DJ628</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>drd</em> Knockdown</td>
<td>No PM (1/18)</td>
<td>No PM (0/13)</td>
<td>PM (14/14)</td>
</tr>
<tr>
<td>Rescue in <em>drd</em> Mutant</td>
<td>PM (17/17)</td>
<td>No PM (0/13)</td>
<td></td>
</tr>
</tbody>
</table>

background during metamorphosis then assaying for the PM 7 days post eclosion. In experiment 1, flies with *drd* knocked down during adulthood for 7 days post eclosion lacked a PM (Table 3.2). This result shows continuous expression of *drd* during adulthood is necessary for PM synthesis. In experiment 2, flies with *drd* knocked down only during metamorphosis had a PM 3 days post eclosion (Table 3.2), providing evidence that *drd* expression during adulthood is sufficient for PM synthesis. In experiment 3, *drd* mutant flies with *drd* rescued during metamorphosis lacked a PM 7 days post eclosion (Table 3.2), providing further evidence that *drd* expression during adulthood is necessary for PM synthesis. Taken together, the results from these 3 temporal experiments suggest *drd* expression during adulthood is necessary and sufficient for adult PM formation.

3.5 Reduced Defecation in *drd* Mutants is Not Due to Lack of a PM

*drd* mutants exhibit reduced defecation rates as adults (Peller et al., 2009). Since *drd* mutants also lack a PM as adults, it is possible the cause of reduced defecation is due to the lack of a PM. To determine if these two
phenotypes are related or independent, I knocked down *drd* only during adulthood so the flies lacked a PM, and measured defecation rate. First, I verified previous findings by measuring defecation rate in *drd* mutant and life-long *drd* knockdown flies. I found results similar to what was previously reported, with a significant reduction in defecation rate of *drd* mutants compared to wildtype flies (Figure 3.3). I then measured defecation rates in flies with *drd*

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Genotype</th>
<th>PM Present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult Knockdown</td>
<td><em>drd</em> Knockdown RNAi #1</td>
<td>0/27</td>
</tr>
<tr>
<td></td>
<td>RNAi #1 Control</td>
<td>19/19</td>
</tr>
<tr>
<td></td>
<td><em>drd</em> Knockdown RNAi construct #2</td>
<td>20/25</td>
</tr>
<tr>
<td></td>
<td>RNAi #2 Control</td>
<td>17/17</td>
</tr>
<tr>
<td>Pupal Knockdown</td>
<td><em>drd</em> Knockdown RNAi #1</td>
<td>13/13</td>
</tr>
<tr>
<td></td>
<td>RNAi #1 Control</td>
<td>15/15</td>
</tr>
<tr>
<td>Pupal Rescue</td>
<td><em>drd</em> Rescue</td>
<td>1/16</td>
</tr>
<tr>
<td></td>
<td>Control Rescue</td>
<td>0/1</td>
</tr>
</tbody>
</table>

*Table 3.2 Continuous Adult Expression of *drd* is Necessary for PM Synthesis*
Figure 3.3 *drd* Mutants Display Reduced Defecation in Early Adulthood

The rate of defecation was measured in 24 and 48 hour old adult male flies. *drd* mutants showed reduced defecation compared to wildtype at both time points (n = 10 groups of two flies, t-test p<.01).

knocked down for seven days starting on the first day of eclosion, which lack a PM based on results from the previous section, and compared to wildtype sibling controls that underwent the same heat shock protocol. Surprisingly, there was no difference in defecation rate between wildtype flies and *drd* knockdowns, suggesting the PM is not necessary for normal adult defecation (Figure 3.4). This result shows the ability to separate the *drd* mutant gut phenotypes of lacking a PM and reduced defecation.
3.6 Discussion

This chapter reported a novel phenotype of *drd* mutants, the lack of an adult PM. More specifically, adult *drd* mutants lack a PM that is visible by gross dissection or chitin staining. The *Drosophila* PM has been shown to consist of at least four distinct layers, not all of which might contain chitin (King 1988, Lehane 1997) and therefore it is possible that *drd* mutants lack only the first layer. In a manuscript published by our lab, histological images of the midgut and cardia show uncondensed material in the cardia that may be components of a PM that fail to crosslink (Conway et al., 2018), but future studies will be necessary to more accurately define the nature of this phenotype.

Interestingly, the larval PM is present in *drd* mutants based on dissection, suggesting that *drd* is necessary for adult PM formation but not necessary for larval PM formation. However, the mechanism underlying why *drd* is necessary for the adult PM but not the larval PM remains unknown, and with the biochemical activity of DRD still undefined, it is difficult to speculate on how *drd* may be functioning differently in larvae versus adults, but is an intriguing avenue of investigation for future studies.

This chapter attempted to answer when and where *drd* expression is necessary for PM formation. A recently published manuscript from our lab shows *drd* to be expressed endogenously at high levels in the anterior cardia, the site of
Figure 3.4 Adult PM is not Necessary for Defecation

The defecation rate of male flies 8 or 9 days post eclosion were measured in wildtype flies and flies with *drd* knocked down only during adulthood. There was no difference in defecation rate between 8-day old wildtype and *drd* adult knockdown flies or 9-day old wildtype and *drd* adult knockdown flies (n = 10 groups of 2 flies, t-test, p>.05).

PM synthesis (Conway et al., 2018). Utilizing the UAS-GAL4 system, I show robust expression of *drd* in the cardia is necessary for PM synthesis as well continuous expression of *drd* in the adult.

Another goal of this chapter was to determine if the lack of a PM is related to or independent of other *drd* mutant phenotypes. It was previously reported
that adult \textit{drd} mutants display reduced defecation (Peller et al., 2009), a result I verified here. However, I was able to separate these two phenotypes through the adult knockdown of \textit{drd}, where flies lack a PM but defecate normally. Additionally, the results of experiments in this chapter combined with preceding work in our lab supports the separation of lacking a PM and early adult lethality. Previous experiments showed knocking down \textit{drd} only during adulthood does not affect survival, while rescuing \textit{drd} during metamorphosis in a \textit{drd} mutant background can rescue survival (Sansone and Blumenthal 2013). I assayed for the presence of the PM under similar experimental conditions, and found flies lacked a PM in both cases while surviving at a wildtype rate, suggesting the lack of a PM and early adult lethality displayed by \textit{drd} mutants are independent phenotypes (Table 3.3).

<table>
<thead>
<tr>
<th></th>
<th>Adult Knockdown</th>
<th>Pupal Knockdown</th>
<th>Pupal Rescue</th>
</tr>
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<tbody>
<tr>
<td>Early Lethality</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Lack PM</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 3.3 Summary of \textit{drd} Survival and Lack of PM Phenotypes

This chapter reports \textit{drd} as necessary for PM synthesis, and \textit{drd} mutants and knockdowns are the first insect lines ever found to completely lack a PM. Finally having an insect that completely lacks a PM will be invaluable for studying PM physiological function, however, \textit{drd} is a far from perfect model. The pleiotropic nature of \textit{drd} makes it difficult to discern experimental results as a
product of lacking a PM vs another *drd* phenotype. While surely *drd* mutants and knockdowns can be used to better understand PM function, it would be ideal to uncover another genetic model that also lacks a PM but without the additional complicating phenotypes of *drd*. 
Chapter 4: multicopper oxidase 4, cyp6t1 and cyp6a22 are Necessary for PM Synthesis

4.1 Introduction

Mutation to the \textit{drd} gene produces flies that lack a PM (Conway et al., 2018), however, use of \textit{drd} mutants to examine PM function would be challenging as they exhibit numerous additional phenotypes such as early adult lethality caused by neurodegeneration and female sterility. Moreover, there are non-PM related gut phenotypes displayed by \textit{drd} mutants, further complicating elucidating the role of the PM with the use of gut specific knockdown of \textit{drd} (Conway et al., 2018). To more accurately define the physiological role of the PM, it would be ideal to identify additional genes that are necessary for PM synthesis that display less pleiotropic effects than \textit{drd} mutants.

In addition to providing a cleaner model to study PM physiological function, identification of genes required for PM synthesis could provide candidate genes for creation of Gal4 and UAS lines with gut or cardia specific expression. A major challenge of understanding \textit{drd} function is the separating the numerous independent phenotypes, but the creation of a GAL4 line with cardia specific expression could allow specific knockdown of \textit{drd} in the site of PM synthesis, which could enable experimentation to more accurately characterize the role of \textit{drd} in PM synthesis. Creation of a UAS line of another gene required for PM synthesis could allow for temporal and spatial expression experiments,
which could potentially shed light on the genetic requirements for PM synthesis as well as provide insights into what genes \textit{drd} may be interacting with biochemically.

A series of RNAi screens assaying for the presence of a PM was done in attempt to identify additional genes required for PM synthesis. After screening 83 RNAi lines, expression in the cardia of three genes, \textit{multicopper oxidase 4} (\textit{mco4}), \textit{cyp6t1} and \textit{cyp6a22}, was found to be necessary for PM synthesis. The biochemical activity of MCO4 was characterized. Finally, to provide tools to better study PM function, there was an attempt to generate \textit{mco4} mutant, \textit{mco4-UAS} and \textit{mco4-gal4} lines of flies.

\textbf{4.2 RNAi Screen of Genes Necessary for PM Synthesis}

Candidate genes were knocked down in the cardia and assayed for the presence of a PM by gross dissection to identify genes necessary for PM synthesis. RNAi was induced in the cardia through \textit{DJ626-gal4} driven expression of RNAi inducing hairpin UAS lines of candidate genes. There were three subgroups of candidate genes 1) genes encoding proteins with known or putative cross-linking activity 2) genes with altered expression in \textit{drd} mutants based on \textit{drd} microarray results 3) lipid processing genes.

Screening for the presence of the PM in twenty lines of UAS induced RNAi against known or putative cross-linking enzymes revealed expression of \textit{mco4} in the cardia to be necessary for PM synthesis (Table 4.1). Screening for the presence of the PM in 38 lines of UAS induced RNAi against candidate genes
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<td>43288</td>
<td>Yes</td>
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**Table 4.1 Crosslinking Enzyme PM Screen**

with altered expression in *drd* knockdowns revealed expression of *cyp6t1* and *cyp6a22* in the cardia is necessary for PM synthesis (Table 4.2). Screening of 28 genes encoding proteins with known or putative lipid processing activity revealed no additional genes necessary for PM synthesis (Table 4.3).
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Table 4.2 *drd* Microarray PM Screen
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**Table 4.3 Lipid Processing PM Screen**
4.3 MCO4 Biochemically Behaves as a Canonical Laccase

Of the four genes found to be necessary for the synthesis of the PM, *mco4* is the most ideal genetic model to study PM function as its expression is localized exclusively to the cardia (flyatlas.org, flygut.epfl.ch, flygut.ord), limiting the likelihood of pleiotropy if the gene is knocked down or mutated, while endogenous expression of *drd, cyp6t1* and *cyp6a22* is found throughout the fly (flyatlas.org, Chung et al., 2009). Based on sequence homology, *mco4* is theorized to encode a laccase, a well conserved family of multicopper-containing oxidizing enzymes necessary for the synthesis of barrier structures in many organisms through a process called sclerotization (Kudanga et al., 2017, Forootanfar et al., 2015). Sclerotization stabilizes insect cuticle through the covalent crosslinking of proteins, which provides rigidity to cuticular structures, such as the exoskeleton and presumably the PM (Dennell 1958, Brunet 1980). The biochemical pathway of sclerotization is well characterized, and typically begins with the introduction of a hydroxyl group into the ortho-position of the phenol group of tyrosine, resulting in the synthesis of dopa, which is further decarboxylated into dopamine by the dopa-decarboxylase family of enzymes (Merrit et al., 1996, Schaefer et al., 1987). Dopamine is then either N-acetylated to form acetyl-D-alanine (NADA) or N-B-alanylated to form NBAD (Mendive 2005). Both NADA and NBAD can be oxidized by laccases to form quinones, which react readily with nucleophilic groups, often protein side chains, that results in the covalent cross-linking of proteins and potentially other nucleophilic compounds, forming cuticular structure (Anderson et al., 1982, Figure 4.1).
To determine if the \textit{mco4} gene product functions like a laccase biochemically, our lab collaborated with Dr. Neal Dittmer, PhD, Research Professor at Kansas State University, to investigate MCO4 activity in vitro. I created a plasmid with the \textit{mco4} CDS followed by a six \textit{his} tag and sent copies of the plasmid to Dr. Dittmer. Dr. Dittmer purified MCO4 and ran a series of analyses to elucidate biochemical function. MCO4 was found to have maximum activity around pH 7 with DMPD pH 6 with NBAD, and additional analysis was conducted at pH 6 (Figure 4.2). Our biochemical analyses of MCO4 displays canonical laccase activity, demonstrated by kinetic assays of MCO4 with p-diphenol compounds DMPD, NBAD, and hydroquinone (Figure 4.3). The lack of a PM in \textit{mco4} knockdown flies suggests \textit{mco4} laccase activity is necessary for PM synthesis, but further in vivo analysis will be needed to determine the endogenous substrates of MCO4 activity.

\textbf{4.4 Construction of \textit{mco4} CRISPR Mutant}

While knockdown of \textit{mco4} with RNAi induced either globally or in the gut can reliably produce larvae and flies lacking a PM, the creation of an \textit{mco4} mutant would provide an incredibly useful tool to study PM physiology and genetic interactions with other genes associated with PM formation. RNAi does not always provide a complete knockdown of a gene and has been shown to occasionally alter expression of off target genes (Moore et al., 2010).
Laccases contain 2 copper cores that facilitate an oxidation reaction. The oxidized substrate can readily form covalent bonds with nucleophilic groups, often protein side chains, that result in cuticle formation.

Figure 4.2 MCO4 has Optimal Activity in Slightly Acidic Environment
MCO4 protein was purified and its activity was measured on various substrates at different pH. (A) MCO4 showed peak activity on DMPD at pH 7. (B) MCO4 showed peak activity NBAD at pH 6.
MCO4 protein was purified and its activity was measured on various substrates canonically associated with laccases. (A-C) MCO4 displayed activity on DMPD, HQ, and NBAD. (D) Summarization of $K_M$, $k_{cat}$ and $k_{cat}/K_M$ of MCO4 across the various substrates.

Additionally, inducing RNAi of two different genes can provide unreliable knockdown of both genes (Haussecker 2016), limiting the certainty of results from potential experiments knocking down multiple genes required for PM synthesis. Thus, the creation of an mco4 mutant line can circumvent these concerns with RNAi and allow for more in depth and reliable experimentation. To generate a mco4 mutant line, I utilized the CRISPR-Cas9 system to mutate the mco4 gene coding sequence (Figure 4.4). I cloned two guide RNA sequences into vectors that would constitutively express the guide RNA upon injection into a
Figure 4.4 *mco4* CRISPR Mutant Generation Model

To generate an *mco4* CRISPR mutant line a backbone plasmid constitutively expressing a guide RNA sequence was injected into a fly line with CAS-9 embedded in its genome. (A) Diagram of guide RNA backbone vector. (B) Cartoon of guide RNA targets, #1 in the third exon and #2 in the fourth exon.
*Drosophila* embryo. One guide RNA sequence targeted the third exon of *mco4* and another sequence targeted the fourth exon of *mco4*. The plasmids were injected independently into embryos of flies that contained germline specific *cas9* expression. After screening through hundreds of flies, 9 potential mutant lines were identified and verified in the flies that had guide RNAs targeting the third exon of *mco4*. One line of mutated *mco4* flies produced a 9bp, three amino acid deletion in the 3rd exon of *mco4* (bps 2123-2131 of the *mco4* CDS) eliminating an ASA amino acid motif. Flies with this mutation lacked a PM (0/20), suggesting the three amino acids that were deleted are necessary for MCO4 to produce a PM. The deletion is not in the predicted catalytic site but rather is embedded in a beta sheet motif of the protein, perhaps indicating these amino acids play an integral role in maintaining a functional conformation of MCO4 necessary for PM synthesis. Since the catalytic sites of MCO4 remain intact, this mutant can not necessarily be characterized as a null mutant, only that this mutant has lost the ability to produce a PM. However, flies from this line were incredibly unhealthy, even when balanced with a *FM7i-gfp* chromosome, providing very few even heterozygous offspring.

**4.5 Construction of mco4-GAL4 Line**

Since *mco4* is expressed highly and nearly exclusively in the cardia and is necessary for the PM, a GAL4 line exhibiting the expression pattern of *mco4* would be valuable tool for experiments examining the genetic requirements for PM synthesis. While there are several published GAL4 lines with various
expression patterns throughout the gut available in multiple stock centers, to our knowledge there is no published GAL4 line with expression limited exclusively to the cardia. The 844bp promoter region of the mco4 gene was cloned into 4 different GAL4 vectors with various degrees of GAL4 expression optimization (Figure 4.5). The four vectors were injected into embryos, however, unfortunately, there were no transformants from any of the injections, even after several attempts.

**Figure 4.5 mco4-GAL4 Generation Model**

To generate an mco4-GAL4 fly line, the mco4 regulatory sequence was inserted in 4 different backbone vectors with varying levels of GAL4 expression.
4.6 Construction of mco4-UAS Line

A mco4-UAS line would be a very useful tool to assess the spatial and temporal expression requirements of mco4 for PM synthesis. To generate a mco4-UAS line of flies, I cloned the CDS of mco4 into a vector with the UAS sequence (Figure 4.6). The vector was injected into embryos, however, after 4 rounds of injections, no embryos matured into adult flies containing the transformation.

4.7 Discussion

The goals of this chapter were to identify additional genes necessary for PM synthesis and develop genetic tools to study PM function. drd is necessary for adult PM synthesis, but due to the pleiotropic nature of drd mutants, it is a poor model to study PM function in isolation. Here, I conducted several RNAi screens and found expression of mco4, cyp6t1 and cyp6a22 in the cardia is necessary for adult PM synthesis.

cyp6t1 and cyp6a22 are cytochrome p450s, but their biochemical role in PM synthesis remains unknown. These genes were identified as candidates due to their altered expression in a drd microarray, but it is not obvious why they would have altered expression in a drd knockdown fly. DRD is a predicted integral membrane acyl-transferase and there is no evidence that it regulates gene expression directly (Blumenthal 2008). It is possible DRD is functioning in
Figure 4.6 *mco4*-UAS Generation Model

To generate an *mco4*-UAS line, the *mco4* cds was inserted into a backbone vector with UAS sites.
a signaling cascade upstream or in parallel of these cytochrome p450s and their altered expression may be the result of compensation or a change in molecular signals in *drd* mutants, however, it is very unclear how this would alter gene expression of *cyp6t1* and *cyp6a22*. Even more confounding is that in *drd* knockdowns *cypt1* is upregulated and *cyp6a22* is downregulated (Blumenthal unpublished). Better understanding of biochemical role of the *drd, cyp6t1* and *cyp6a22* gene products could perhaps allow for a more refined hypothesis on how these genes are interacting and why they are all necessary for adult PM formation.

*mco4* is the cleanest genetic model to study PM physiological function as its expression is localized to the cardia, while the other three genes are expressed in additional tissues, inviting the possibility of pleiotropy in PM studies. In collaboration with Dr. Neal Dittmer, PhD, Research Professor at Kansas State University, we showed MCO4 to exhibit canonical laccase activity. This result is unsurprising but reassuring, as sequence homology from *mco4* cds predicted a laccase gene product. MCO4 behaving as a laccase also fits seamlessly with canonical functionality of laccases across many organisms, as this family of proteins is essential in cuticular barrier structure crosslinking, and knockdown of *mco4* produces flies that lack a PM, a cuticular barrier structure. While MCO4 has activity on common laccase substrates, it remains unknown what substrates specifically MCO4 uses in vivo, and future studies will be needed to elucidate natural MCO4 substrates. Also unsurprising is the observation that MCO4
functions optimally around pH 6, as the cardia has been shown to be a slightly acidic environment and mco4 is expressed nearly exclusively in the cardia (Shanbhag et al., 2009, flyatlas.org).

I attempted to create mco4 based genetic tools to allow for more in-depth study of PM physiological function and synthesis requirements. Using CRISPR technology, I created an mco4 mutant line with a 3 amino acid deletion in the third exon. While I was hopeful this line would be fruitful in a multitude of potential experiments addressing PM function, the line was unhealthy, yielding an incredibly low number of offspring, most of which would die within a week of eclosion, even when the genetic background was backcrossed against wildtype flies and the mutated chromosome was placed over a balancer. The health of this mutant line was very surprising because RNAi knockdown of mco4 either globally or in a gut specific manner has no major effect on fecundity. Why the mutant flies were so much unhealthier compared to RNAi knockdown flies remains a mystery, but the low fecundity of this line limits the experimental potential. It is possible mco4 is essential in early embryonic development and RNAi is not induced early enough to cause lethality while mutation to mco4 induces early lethality. I screened over 400 flies while assaying for mco4 mutants and only one line that survived to adulthood had a mutation to mco4, therefore, it may be an essential gene. CRISPR is very robust in creating mutants, and if mco4 was not essential for survival there would likely be a much higher number of mutants. Perhaps the nature of this mutant line can suggest something about mco4 functionality, that the three amino acids deleted are
necessary for PM formation but not completely necessary for survival. Additionally, MCO4 functions as a dimer (Dittmer unpublished), and the small number of heterozygous mco4 CRISPR mutants may suggest that this mutant is a dominant negative. Further studies will be needed to address why mco4 mutants are so much less healthy than knockdowns.

I also attempted to created mco4-Gal4 and mco4-UAS lines. While successfully engineering four mco4-Gal4 and a mco4-UAS plasmids, and despite using injection companies and backbone plasmids that have yielded transformants for our lab in the past, there were no adult transformant flies for any of the vectors. This result was surprising, as it is difficult to pinpoint why these vectors could not be transformed. There was hope that an mco4-Gal4 line would produce a cardia specific driver that would allow for precise spatial expression for a number of genetic experiments, including cardia specific expression of drd to further characterize drd PM and non-PM related gut phenotypes. It remains unknown why these lines could not be created from injection of these vectors.

Despite the lack of success creating useful genetic tools, it will still be possible to use mco4 knockdowns as a powerful instrument to define PM physiological function. For experimental convenience and further verification, it would have been helpful to have a healthy mco4 mutant line, but nearly any experiment examining PM function can still be conducted using mco4 RNAi knockdowns, which consistently produce flies that lack a PM. Ideally, if the mco4 UAS and Gal4 lines were created, there would be a multitude of intriguing
genetic expression experiments to better define the requirements for PM synthesis, but there are still Gal4 lines that can drive strong expression in the cardia available for experimentation, just not as exclusively as was hoped by a potential mco4-gal4.
Chapter 5: cyp6a22 and cyp6t1 Knockdowns Display drd Mutant Gut Phenotypes but Do Not Display Non-Gut drd Mutant Phenotypes

5.1 Introduction

An RNAi screen of genes showing a significant change in expression in drd knockdowns revealed two cytochrome p450 genes that are essential for PM formation: cyp6a22 and cyp6t. Cytochrome p450s are hemoproteins, meaning they contain a ferrous binding site and typically require an iron cofactor for activity (Estabrook 2003). The Drosophila melanogaster genome contains 90 cytochrome p450 genes, most of which have undetermined function (Good et al., 2014). All studied cytochrome p450 genes whose function has been analyzed show the ability to metabolize either exogenous or endogenous substrates, and have therefore been linked to hormone and signaling pathways in addition to their most commonly studied function, insecticide resistance (Bass et al., 2015, Balabanidou et al., 2016). A large portion of cytochrome p450 family members are found in the midgut, where they metabolize potentially harmful chemicals into substances that will not damage the fly, hence why they are studied in the context of insecticide resistance. The function of neither cyp6a22 nor cyp6t have been examined.

In this chapter I examine the expression requirements of cyp6a22 and cyp6t1 for adult and larval PM synthesis. Additionally, since cyp6a22 and cyp6t1 were identified as candidate genes from a drd microarray where they displayed altered expression in drd knockdowns, it is possible they are functioning in similar
genetic pathways. This chapter examined if \textit{cyp6a22} and \textit{cyp6t1} knockdowns phenocopy \textit{drd} knockdowns in both gut and non-gut deficiencies.

5.2 \textit{cyp6a22} and \textit{cyp6t1} Larval Knockdowns Have a PM but Adult Knockdowns Lack a PM

\textit{cyp6a22} and \textit{cyp6t1} were identified as necessary for PM synthesis via a screen by gross dissection of the adult midgut. To confirm the absence of a PM in adult knockdown flies, dissected midguts were stained with the chitin binding fluorophore calcofluor. Both \textit{cyp6a22} and \textit{cyp6t1} adult knockdown flies exhibit a weak, diffuse staining of calcofluor while wildtype parental controls show a clear, bright staining of the PM (Figure 5.1, N=5 per genotype). These images confirm the absence of the PM in \textit{cyp6a22} and \textit{cyp6t1} adult knockdowns. While adult knockdowns of \textit{cyp6a22} and \textit{cyp6t1} lack a PM, larval knockdowns of \textit{cyp6a22} and \textit{cyp6t1} still had a PM (12/12 for each genotype).

5.3 \textit{cyp6a22} and \textit{cyp6t1} Knockdowns are Fertile and Display Normal Survival

\textit{cyp6a22} and \textit{cyp6t1} have altered expression in \textit{drd} knockdowns and like \textit{drd} knockdowns lack a PM as adults but not as larvae. Therefore, it is possible \textit{drd} and \textit{cyp6a22} and \textit{cyp6t1} may be functioning in similar genetic pathways. To gather evidence if \textit{cyp6a22}, \textit{cyp6t1} and \textit{drd} are functioning in similar non-gut
pathways, I examined the role of cyp6a22 and cyp6t1 on female fertility and survival; 2 major adult phenotypes of drd mutants. cyp6a22 and cyp6t1 were knocked down via RNAi driven by the ubiquitous DA-gal4 line and assayed for fertility by crossing knockdown flies with wildtype flies from a parental line. Both male and female cyp6a22 and cyp6t1 knockdowns were fertile (Table 5.1). It was previously reported drd mutant and knockdown female flies are sterile (Blumenthal 2008), therefore, these results suggest, unlike drd, cyp6a22 and cyp6t1 are not necessary for female fertility.

drd mutant flies display an acute early adult lethality phenotype. cyp6a22 and cyp6t1 knockdowns were scored for survival over 40 days to examine if their expression is also necessary for adult survival. cyp6a22 and cyp6t1 knockdowns did not display a drd early adult lethality phenotype, in fact, cyp6a22 and cyp6t1
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Table 5.1 *cyp6a22* and *cyp6t1* Knockdowns are Fertile

**Figure 5.2 cyp6a22 and cyp6t1 Knockdowns Survive at Wildtype Rate**

Flies were assayed for survival over 40 days. (A) *drd* mutants’ median life span was 5 days while ubiquitous rescue of *drd* in a *drd* mutant background can rescue survival (From Sansone dissertation 2013). (B) *cyp6a22* and *cyp6t1* knockdowns survive at a wildtype rate (N= 50 flies per genotype, Mantel-Cox Survival Test p>0.05).
knockdowns survive at a wildtype rate over 40 days, suggesting neither \textit{cyp6a22} nor \textit{cyp6t1} is necessary for adult survival over 40 days (Figure 5.2).

While \textit{cyp6a22} and \textit{cyp6t1} adult knockdowns do not phenocopy \textit{drd} mutants in terms of female sterility and early adult lethality, I can not rule out that the cytochrome p450s may be interacting in these pathways with \textit{drd}, only that both \textit{cyp6a22} and \textit{cyp6t1} are not necessary for female fertility and adult survival.

\textbf{5.4 \textit{cyp6a22} and \textit{cyp6t1} Knockdowns Display Early Adult Immune Activation and Decreased Defecation}

In addition to lacking a PM, \textit{drd} mutants display gut dysfunction phenotypes of early adult activation of the \textit{IMD} innate immunity pathway as well as reduced defecation (Peller et al., 2009, Blumenthal unpublished). The \textit{IMD} pathway is typically induced upon infection of gram negative bacteria in the lumen of the \textit{Drosophila} midgut and the level of induction can be measured by the expression of the antimicrobial peptide \textit{diptericin (dpt)} via qPCR (Hanson et al., 2016). Previous unpublished work in our lab showed \textit{drd} mutant flies display a striking expression of \textit{dpt} in two-day old male flies compared to wildtype, a result I replicated here (Figure 5.3). To determine if \textit{cyp6a22} and \textit{cyp6t1} phenocopy the \textit{drd} mutant early immune activation phenotype, the level of \textit{dpt} in \textit{cyp6a22} and \textit{cyp6t1} knockdowns was measured and found to be prominently higher in the knockdowns compared to wildtype as well (Figure 5.3). This result suggests \textit{cyp6a22} and \textit{cyp6t1} are necessary for wildtype immune activation.
Figure 5.3 *drd* Mutants and *cyp6a22* and *cyp6t1* Knockdowns Display Early Adulthood Immune Activation

The relative expression of the antimicrobial peptide *dpt* was measured in two-day old adult flies via qPCR. *drd* mutants and *cyp6a22* and *cyp6t1* knockdowns display a significant increase in immune response compared to wildtype (ANOVA, Tukey Post Hoc, p<0.01, N=3 replicates of 10 flies per genotype).

I confirmed in an earlier chapter the observation that *drd* mutants display reduced adult defecation (Peller et al., 2009). To determine if *cyp6a22* and *cyp6t1* are also necessary for defecation rate, I measured the rate of defecation of two-day old male *cyp6a22* and *cyp6t1* knockdown flies. *cyp6a22* and *cyp6t1* knockdown flies display a reduced rate of defecation compared to wildtype flies, however, both knockdowns defecate at a significantly higher rate than *drd* mutant.
flies (Figure 5.4, Figure 3.3). This result suggests \textit{cyp6a22} and \textit{cyp6t1} are necessary for wildtype defecation rate.

\textbf{5.5 Discussion}

Expression of \textit{cyp6a22} and \textit{cyp6t1} in the cardia was found to be necessary for PM synthesis in an RNAi screen, and this chapter aims to determine additional gut and non-gut phenotypes of \textit{cyp6a22} and \textit{cyp6t1} knockdowns. Specifically, the goal of this chapter was to determine how closely \textit{cyp6a22} and \textit{cyp6t1} knockdowns phenocopy \textit{drd} mutants and knockdowns. The screen that yielded \textit{cyp6a22} and \textit{cyp6t1} as necessary for PM synthesis was based on candidate gene families that displayed altered expression in a \textit{drd} microarray in stage 10 egg chambers (Blumenthal unpublished). Since knocking down \textit{cyp6a22}, \textit{cyp6t1} or \textit{drd} causes flies to lack a PM and \textit{cyp6a22} and \textit{cyp6t1} display altered expression in at least one tissue type of \textit{drd} knockdowns, they may be interacting in similar genetic pathways. Based on results from this chapter, unlike \textit{drd}, \textit{cyp6a22} and \textit{cyp6t1} are not necessary for adult survival or female fertility, but, like \textit{drd}, \textit{cyp6a22} and \textit{cyp6t1} knockdowns display early adult immune activation and reduced adult defecation.

Taken together, the results from this chapter suggest \textit{cyp6a22} and \textit{cyp6t1} knockdowns phenocopy \textit{drd} in a gut specific manner. A previous study characterized the expression profile of numerous \textit{Drosophila} cytochrome p450s in larva and adults and \textit{cyp6a22} and \textit{cyp6t1} both show strong expression in the larval and adult midgut and fat body, with mild expression in various other tissues.
Figure 5.4 *cyp6a22* and *cyp6t1* Knockdowns Display Reduced Defecation

Adult flies were assayed for defecation rates at 24 and 48 hours post eclosion. *cyp6a22* and *cyp6t1* knockdown flies showed reduced defecation at both 24 and 48 hours (ANOVA, Tukey Post Hoc, p<0.01, N=20 per genotype).

(Chung et al., 2009). The biochemical activity of *cyp6a22* and *cyp6t1* specifically has not been elucidated, but numerous studies have implicated many *Drosophila* cytochrome p450s as necessary for hormone signaling in various tissues for a multitude of developmental pathways (Good et al., 2014, Markov et al., 2009, Sztal et al., 2012). It is possible that *cyp6a22* and *cyp6t1* may function in the same genetic pathway as *drd* for PM synthesis but not functioning in the same pathway as *drd* for female fertility and neuronal development. However, while *cyp6a22* and *cyp6t1* knockdowns did not phenocopy the non-gut *drd* mutant phenotypes, I can not rule out they are not functioning in those pathways completely, just that they are not necessary for survival or female fertility. Future
studies will be needed to determine if \textit{cyp6a22} and \textit{cyp6t1} function in additional \textit{drd} pathways and if they are interacting with \textit{drd} directly.
Chapter 6: *mco4* is Necessary for Larval PM and Optimal Digestive Efficiency

6.1 Introduction

The primary function of the animal gut is to digest food and absorb nutrients, processes that can be influenced by a multitude of factors including secretion of digestive enzymes, contraction of gut muscles, microbiome composition, hormonal signaling and stage of organismal development (Broderick et al., 2012, Buchon et al., 2013, Combe et al., 2014, Kannan and Fridell, 2013, Mirth et al., 2012). As food moves through the animal gut, food is mechanically degraded and moved by muscular contractions through defined regions with varying pH containing digestive enzymes responsible for breaking down macromolecules into smaller components which are then absorbed by specialized cells (Droujinine et al., 2016, Karasov and Douglas, 2013, Sarov et al., 2016).

Defects in nutritional uptake can lead to developmental issues for many organisms (Wang et al., 2012, Liu et al., 2016). In *Drosophila*, the most nutritionally sensitive developmental time point is the third instar larval stage (L3), when the larva is constantly eating and gaining mass at a rapid rate to prepare for metamorphosis (Silva-Soares et al., 2017). The length of L3 and rate of weight gain during L3 are directly related to digestion, as the length of L3 can be extended and rate of growth slowed if there are digestive inefficiencies (Chu et al., 2014). These changes in nutritional uptake can influence insulin signaling,
well characterized patterns responsible for organismal development (Ikeya et al., 2002). Some *dilps* are influenced by nutritional uptake while other factors are independent of nutrition, providing a reliable readout on the type of developmental stress a larva may be undergoing (Kannan and Fridell, 2013).

The *Drosophila* PM is theorized to modulate several gut processes and play an integral role in digestion (Hegedus 2009, Kuraishi et al., 2011). However, to date, all published studies investigating the role of the *Drosophila*, or any other insect, PM in digestion utilize lines with PMs that have been partially damaged either pharmacologically or genetically, but never with the complete absence of a PM, limiting the depth of investigation (Kuraishi et al., 2013). While some of these “leaky” PM models show digestive defects, the mechanism of how the PM is serving a digestive function remains unknown.

As reported in previous chapters, *mco4* knockdown larvae completely lack a PM, and are an ideal tool for studying PM physiological function due to the lack of pleiotropy. In this chapter, *mco4* knockdown larvae are utilized to better characterize the role of the PM in larval digestion and development, as well as define a mechanism for how the PM may influence digestive processes.

6.2 Expression of *mco4* in the Cardia is Necessary for PM Formation

Expression of *mco4* in the cardia was found to be necessary for PM formation via an RNAi screen and gross dissection (Chapter 4). To confirm the absence of the PM, I dissected larval and adult guts and incubated with a chitin binding fluorophore, Calcofluor, bath. Wildtype guts (Dagal4, DJ626, UAS-
mco4RNAi) showed a bright, structured fluorescence lining the inside of the midgut indicating the presence of a PM, while mco4 knock down larvae and flies show a very weak, diffuse staining, indicating the absence of a PM (Figure 6.1). Therefore, expression of mco4 in the cardia is necessary for PM formation in both larvae and adult flies.

6.3 Larval PM is Necessary for Normal Weight Gain and Developmental Timing

*Drosophila* adult body mass is determined during the larval stages of development and deficiencies in nutritional uptake results in smaller flies and a prolonged L3 stage (Handke 2013, Mirth 2012). *mco4* knockdown flies displayed a smaller adult body mass than wildtype flies (knockdown females weigh 72.7% of wildtype, knockdown males weigh 78.2% wildtype Figure 6.2). The L3 stage is longer in *mco4* knockdown larvae compared to wildtype parental controls (female knockdown rate 124.7% wildtype, male knockdown 122.3% wildtype, Figure 6.3). *mco4* knockdown larvae also gain weight at a slower rate in L3 compared to wildtype parental controls (Figure 6.4). These results suggest the larval PM is necessary for normal development.
Figure 6.1 *mco4* Knockdowns Lack a PM

Dissected larval and adult fly guts were incubated in the chitin binding fluorophore calcofluor to assay for the presence of a PM. The presence of a PM was defined as a bright, organized illumination of the structure. Ubiquitous (*Da-gal4*) and gut specific (*DJ626*) knockdown of *mco4* eliminates the PM in both larvae and adult flies (n= 5 per genotype).
Figure 6.2 Reduced Adult Body Mass in *mco4* Knockdowns

Two-day old flies were weighed. *mco4* knockdown males and females weighed less than wildtype flies (n=23-39 per genotype. ANOVA, Tukey Post Hoc, p<0.01).

Figure 6.3 L3 Extended in *mco4* Knockdowns

The length of L3 was measured in larvae. *mco4* knockdown larvae have an extended L3 compared to wildtype (n= 31-42 per genotype. ANOVA, Tukey Post Hoc, p<0.01).
Figure 6.4 Lower Rate of Weight Gain in mco4 Knockdown Larvae

Larvae were weighed at various points in L3. In both males and females mco4 knockdown larvae gain weight at a lower rate than wildtype (n=12-19 per group, regression analysis p<0.05).
6.4 Developmental Delay in mco4 Knockdowns is Due to Nutritional Uptake Defect

Nutrition is a primary regulator of larval development and here we tested if larvae lacking a PM display a development phenotype due to a lack of nutrients (Layalle et al., 2008). Expression patterns of various Drosophila insulin like peptides (dilps) are influenced by different environmental cues (Garelli et al., 2012, Uryu et al., 2013).

To determine if the developmental defects experienced by larvae lacking a PM was due to nutrition, I measured the levels of several dilps at various time points throughout development. Whole fly dilp2 expression levels are influenced throughout development by oxygen and light, but not nutrition (Ikeya et al., 2002, Kannan and Fridell, 2013). The relative amount of dilp2 was unchanged throughout development in both mco4 knockdown larva and wildtype parental controls, suggesting any developmental deficiency was not due to oxygen availability or irregular light exposure (Figure 6.5). dilp3 and dilp5 expression drops in larvae that are starved (Ikeya et al., 2002, Kannan and Fridell, 2013). dilp3 expression is significantly lower in mco4 knockdowns compared to wildtype controls at similar time points (Figure 6.6A). dilp5 expression is significantly lower in mco4 knockdowns compared to wildtype larva 24 hours into L3, but there is no significant difference between mco4 knockdowns and wildtype larvae at 46 hours into L3 nor do the 58 hour into L3 mco4 knockdowns exhibit a
Figure 6.5 Larval *dilp2* Expression Unchanged in *mco4* Knockdowns

The relative levels of *dilp2* were measured at various points in L3. There was no significant change in expression between any of the time points (n=3 replicates of 10 larvae per group, ANOVA, p>0.05).
Figure 6.6 Larval dilp3 and dilp5 Expression Lower in mco4 Knockdown Larvae

The relative levels of dilp3 and dilp5 were measured at various points in L3. (A) dilp3 levels were lower at 24 and 46 hours in mco4 knockdown larvae compared to wildtype (N=3 replicates of 10 larvae per group, ANOVA, Tukey Post Hoc, p<0.05). (B) dilp5 levels were not different between mco4 knockdowns and wildtype at 24 hours into L3 (n=3 replicates of 10 larvae per group, ANOVA, p>0.05) but at 46 hours into L3 mco4 knockdowns displayed significantly lower levels of dilp5 (n=3 replicates of 10 larvae per genotype, ANOVA, Tukey Post Hoc, p<0.05).

...significant decrease in expression compared to any wildtype time point (Figure 6.6B). dilp8 inhibits the synthesis of ecdysone, the primary signal for initiating the transition from larva to pupation and remains at a high expression level until the larva has acquired enough nutritional stores to transition to metamorphosis, when its expression then drops dramatically (Garelli et al., 2012, Uryu et al.,...
Wildtype and mco4 knockdown larvae maintain a high level of dilp8 expression 24 hours into L3 as expected, but at 46 hours into L3, wildtype flies show a striking decrease in dilp8 expression while mco4 knockdown larvae maintain a constant level of expression and do not experience a drop in expression until 58 hours into L3, suggesting mco4 knockdown larvae are acquiring nutrients at a slower rate than wildtype flies (Figure 6.7). Taken together, the expression profiles of various dilps suggest the development defects experienced by larvae lacking a PM is due to starvation.

6.4 Lacking a Larval PM has No Effect on Food Movement in the Gut

While it appears mco4 knockdown larvae are suffering from mild starvation, this phenotype could result from a number of different defects in gut function. One possibility is that the PM is necessary for normal movement of food through the gut. To test this hypothesis, we quantified the amount of food L3 larvae consume and defecate. I first measured larval gut contractions and there was no difference between mco4 knockdowns and wildtype parental controls (Figure 6.8). mco4 knockdown larvae consume less food in L3 than wildtype parental controls, but when the amount of food per larva is normalized to their body mass, there is no difference in consumption between wildtype and mco4 knockdown larvae (Figure 6.9). mco4 knockdown larvae defecate less than wildtype parental controls at the same age in L3, but when defecation amount is normalized for larval body mass, there is no significant difference between the knockdowns and
The relative levels of *dilp8* were measured via qPCR at various points in L3. At 24 hours into L3 there was no difference in *dilp8* expression between *mco4* knockdowns and wildtype larvae (n=3 replicates of 10 larvae per group, ANOVA, p>0.05) but there was a significant difference in *dilp8* expression 46 hours into L3 between *mco4* knockdown and wildtype larvae (n=3 replicates of 10 larvae per group, ANOVA, Tukey Post Hoc, p<0.05).

**Figure 6.7 dilp8 Drop Delayed in mco4 Knockdown Larvae**
Figure 6.8 Gut Contraction Rate Normal in mco4 Knockdown Larva

The rate of gut contractions per two minutes was measured in 3 distinct gut regions, the anterior midgut, the acidic region and the posterior midgut. There were no significant differences between mco4 knockdown larva and wildtype rates at any corresponding gut regions (n= 17-24 per genotype, ANOVA p>0.05).
Figure 6.9 mco4 Knockdown Larvae Consume Food at a Wildtype Rate When Normalized for Body Mass

Larvae were placed on dishes with blue dyed food for one hour and the amount of food consumed was measured by light spectrophotometry. (A) mco4 knockdown larvae consumed less total food than wildtype (n= 50 per genotype, ANOVA, Tukey Post Hoc, p<0.05). (B) When the amount of food consumed was normalized for the weight of the larvae, there was no difference between mco4 knockdown larvae and wildtype (n=50 per genotype, ANOVA p>0.05).

controls (Figure 6.10). These results suggest lacking a larval PM has no effect on the movement of food in the larval gut.

6.5 Raising mco4 Knockdown Flies on a More Nutritious Food Source Can Partially Rescue the Mild Starvation Phenotype

It is possible larvae lacking a PM may be suffering from a digestion or nutrient absorption defect. To test this theory, I raised flies on Nutri-Fly
Figure 6.10  mco4 Knockdown Larvae Defecate at Wildtype Rate When Normalized for Body Mass

Larvae were fed blue dyed food for 1 hour then transferred to a sucrose plate for 2 hours. The plates were homogenized and the amount of food defecated was quantified with photo spectrometry. (A) mco4 knockdown larvae defecated less total food than wildtype (n= 50 per genotype, ANOVA, Tukey Post Hoc, p<0.05). (B) When the amount of food defecated was normalized for the weight of the larva, there was no difference between mco4 knockdown larvae and wildtype (n=50 per genotype, ANOVA p>0.05).
Figure 6.11 Body Mass Partially Rescued in *mco4* Knockdown Flies Raised on More Nutritious Food

Flies were raised on the more nutritious Nutri-Fly™ “German Food” Sick Fly Formulation then weighed as 2-day old adults. (A) In both females and males, *mco4* knockdown flies weigh significantly less than parental wildtype controls (ANOVA, Tukey post hoc test, p<0.05, n= 23-29 per sex and genotype). (B-C) The percent increase in body mass from flies raised on normal food (Figure 6.2) compared to flies raised on Nutri-Fly™ “German Food” Sick Fly Formulation was scored per genotype. In both females and males, wildtype flies showed a modest increase in body mass (Da-gal4: females 114.1%, males 110.6%, UASmco4RNAi: females 102.3%, males 115.9%) while *mco4* knockdown flies show a pronounced increase in body mass (females 136.8%, males 123.5%).
“German Food” Sick Fly Formulation in attempt to rescue the mild starvation phenotype. The “German Food” is a much more nutrient and calorically rich media that requires less hydrolysis as it contains more individual amino acids compared to normal laboratory food. I quantified a rescue of the mild starvation phenotype by measuring adult mass. All genotypes, for both sexes, raised on the more nutritious food showed an increase in body mass compared to normal laboratory food (Figure 6.11). Despite this increase, mco4 knockdowns were still significantly smaller than their parental controls when raised on nutritious food. However, mco4 knockdowns showed a significantly larger increase in body weight between the food sources compared to wildtype genotypes (Da-gal4: females 114.1%, males 110.6%, UASmco4RNAi: females 102.3%, males 115.9%, mco4 knockdown females 136.8%, males 123.5%). Therefore, it appears the more nutritious food has a greater effect on mco4 knockdowns, suggesting a partial rescue of the starvation phenotype.

6.6 Discussion

Since mco4 is expressed highly and nearly exclusively in the cardia according to several online gene expression databases (flyatlas.org, flygut.epfl.ch), it is an ideal gene knockdown candidate to study PM physiological function with potentially minimal pleiotropic effects. One major phenotype of lacking a PM is reduced adult body weight (Figure 6.2). We show this reduced body weight can be attributed to an extended L3 stage (Figure 6.3) and slower weight gain during L3 (Figure 6.4) in mco4 knockdown larva. Several factors can
influence *Drosophila* development in L3 including light cycles and oxygen levels, but the most likely cause for this delay in mco4 knockdowns is mild starvation. We showed mco4 knockdown larvae exhibit a starvation dilp profile. Previous studies have stated starved larvae show decreased in dilp3 and dilp5 expression, but maintain a wildtype level of dilp2 expression (Ikeya et al., 2002, Kannan and Fridell, 2013). Our results showed a similar pattern with the only incongruity being a similar level of dilp5 expression in wildtype and mco4 knockdown larvae at 46 hours into L3 (Figure 6.6, 6.6). While surprising, this incongruity may give credence to the theory that mco4 knockdowns are experiencing only a mild starvation phenotype. I also measured dilp8 expression, which is nutrient sensitive and inhibits the release of ecdysone, the primary signal for the larval to pupal transition (Garelli et al., 2012, Uryu et al., 2013). In agreement with previous literature, wildtype larvae experience a sharp decrease in dilp8 expression at 46 hours into L3 while mco4 knockdown larvae still maintain a high level of dilp8 expression at 46 hours into L3 and do not see a large drop until 58 hours into L3, signifying mco4 knockdowns are reaching the nutritional uptake threshold to transition to pupation at a slower rate (Figure 6.7). Taken together, these results show mco4 knockdown larvae are experiencing a starvation phenotype. It is noteworthy that the developmental delay experienced by mco4 knockdowns is about 12 hours and previous studies have shown flies can be starved enough to extend L3 by about 24 hours and still be viable adults (Handke et al., 2013, Mirth et al., 2012), suggesting mco4 knockdowns may be experiencing a mild starvation phenotype.
Larvae lacking a PM appear to have a defect in food digestion or nutrient absorption. I can rule out a food movement inefficiency as the cause of mild starvation as there was no difference between wildtype and mco4 knockdown larvae in gut contraction, food consumption or defecation (Figure 6.8, 6.9, 6.10). However, when larvae were raised on a more nutritious food source, mco4 knockdown flies show a much greater percentage weight increase between the food sources compared to wildtype flies (Figure 6.11). While raising flies without a PM on more nutritious food may not totally rescue the starvation phenotype, there is a mitigation of the severity of the phenotype, suggesting the larval PM plays a role in digestion or nutrient absorption.

Future research will need to address the mechanism for the mild starvation experienced by mco4 knockdown larvae as several processes integral for digestion and nutrient absorption could be affected by the lack of a PM. Numerous studies show the impactful role of residual microbes on digestion (Broderick et al., 2012, Combe et al., 2014, Wong et al., 2016) and it is possible lacking a PM may affect microbial load, composition or function. Compartmentalization of distinct pH regions throughout the midgut is essential for digestive function (Shanbhag et al., 2009, Overend et al., 2016) and could be affected by the lack of a PM. Additionally, the activity or secretion of critical digestive enzymes like lipases or proteases (Zinke et al, 2002, Nirala et al., 2013, Buchon et al., 2013) may be affected by the absence of the PM. Despite not knowing the exact mechanism of how the larval PM may influence digestion, it
clearly does, and future scientific or medical studies investigating digestive function should consider the role of barrier structures.
Chapter 7: The Peritrophic Matrix is Necessary for Maintaining Adult Microbiome and Gut Homeostasis

7.1 Introduction

The balance of maintaining a stable microbiome while protecting the gut from pathogenic infection is essential for gut homeostasis and organismal fitness (Sleiman 2015). Commensal bacterial populations play integral roles in digestion, pathogen defense and stem cell turnover (Broderick et al., 2012, Fink et al., 2013, Bost et al., 2017). Ablation or augmentation of commensal microbial diversity can lead to changes in host transcriptional regulation, life span, reproductive success and metabolism (Lizé et al., 2014, Yuval 2017). Most animals have an innate immune system, typically inducible by bacteria, in their gut to protect the organism from infection, along with well-defined pH regions (Lee and Lee 2013). Investigating how a commensal bacteria population can maintain a stable population and diversity in such a dynamic environment is a growing area of interest (Mistry and Kou 2017).

In *Drosophila*, numerous studies have characterized the robust relationships between commensal bacterial and immune response pathways. Two major innate immunity pathways, *imd* and *Toll*, are activated upon bacterial infection of the gut (Buchon et al., 2013). Commensal bacterial populations reduce innate immune induction and ablation of the microbiome leads to an exaggerated immune response upon infection of pathogenic bacteria (Gilbert et al., 2014). The typical laboratory raised *Drosophila* gut microbiome contains 6-
12 families of bacteria with *Acetobacter* (60-80%) and *Lactobacillus* (10-20%) dominating the total abundance (Early et al., 2017). Changes to microbial population abundance or diversity can induce immune pathway responses (Zhai et al., 2017).

*Drosophila* continues to emerge as a leading model for studying host-microbiome interactions due to the simplicity of the laboratory raised microbial diversity and the technical ease of experimental manipulation (Fischer et al., 2017). Despite significant progress in defining host-microbiome relationships, there has been little work done to elucidate the role of barrier structures in these interactions. It remains nearly entirely unknown how the PM may influence commensal bacteria and immune interactions. In this chapter, with the use of *mco4* knockdown flies, I investigate how the PM augments host-microbiome interactions and how these interactions influence host fitness.

**7.2 mco4 is Not Necessary for Gut pH Compartmentalization**

Compartmentalization of distinct pH regions throughout the midgut is essential for digestive function in many organisms and can influence microbial populations (Shanbhag et al., 2009). Digestive enzymes function and commensal bacteria grows optimally in specific and unique pH ranges while the process of water reabsorption near the end of the animal digestive tract is also dependent on pH (Overend et al., 2016). The *Drosophila* gut has a well-defined pattern of pH compartmentalization, with a highly acidic region in the anterior
midgut followed by a gradual increase in pH through the midgut to near neutral levels, and finally an alkaline region throughout the hindgut (Buchon et al., 2015).

Since the PM is semi-permeable and lines nearly the entire digestive tract, it may play a role in pH compartmentalization. Adult flies were fed a solution of sucrose and Sigma m-Cresol purple pH dye, which displays different colors in various pH ranges, and their guts were dissected to visualize any changes in pH throughout the gut (Overend et al., 2016). *mco4* knockdowns and wildtype flies showed a similar pattern of pH compartmentalization as reported in the literature, suggesting the PM is not necessary for pH compartmentalization (Figure 7.1). Therefore, it is likely any changes in microbial communities or immune activation observed in early adult *mco4* knockdowns are not influenced by a change in pH.

### 7.3 *mco4* is Not Necessary for Early Adult Defecation

The rate of food movement in the gut can influence microbial community load, and therefore immune response levels (Blum et al., 2013). Findings in a previous chapter showed reduced defecation in *drd* mutants and *cyp6t1* and *cyp6a22* knockdown adults, suggesting the PM is necessary for adult defecation. However, another previously described experiment knocking down *drd* only during adulthood eliminates the PM and has no effect on defecation, suggesting the PM is not necessary for adult defecation. Since the *mco4* knockdown model is the cleanest method for eliminating the PM, *mco4* knockdown adult flies were scored for adult defecation. In two-day old adult male flies, there was no difference in defecation rate between wildtype and *mco4* knockdown flies (Figure
Flies were fed a dye that displays different colors at particular pH ranges and their guts were visualized under a dissecting scope (50x magnification). *mco4* knockdown and wildtype flies display a similar pattern of pH compartmentalization. Region 1 is highly acidic indicated by yellow coloring. Region 2 is slightly acidic based on clear coloring. Region 3 is slightly alkaline based on purple staining (n=10 per genotype).

This result, in addition to the *drd* adult specific knockdown experiment, suggest the adult PM itself is not necessary for normal adult defecation rate. Therefore, it is likely any observed changes in early adulthood immune activation or microbial load in *mco4* knockdowns will not be due to food movement rate.

**7.4 Knockdown of *mco4* Does Not Induce Early Adult Immune Response**

Previous chapters showed an upregulation of the *IMD* pathway antimicrobial peptide *diptericin (dpt)* in 2-day old adult male flies in *drd* mutants and *cyp6t1* and *cyp6a22* knockdowns. It is possible the PM is necessary for early adulthood immunity and when eliminated, there is an upregulation of the *IMD* pathway to compensate. This hypothesis was tested by measuring *dpt* in 2-day old *mco4* knockdown male flies. Unlike *drd* mutants and *cyp6t1* and
Adult flies were assayed for defecation rates at 24 and 48 hours post eclosion. mco4 knockdown flies exhibited wildtype defecation rates at both 24 and 48 hours post eclosion (n=24-28 per genotype, t-test, p>0.05).

cyp6a22 knockdowns, mco4 knockdown flies showed a wildtype level of dpt expression (Figure 7.3). This result suggests the PM itself is not necessary for immunity in early adults.

7.5 mco4 Knockdowns Display Wildtype Early Adult Gut Microbial Load

The PM is theorized to aid in maintenance of the gut microbiome. To examine the role of the PM in early adult microbial load, the relative levels of bacteria via 16S ribosomal subunit abundance was measured by qPCR. In 2-
Figure 7.3 *mco4* Knockdowns Do Not Display Early Adulthood Immune Activation

The relative level of the antimicrobial peptide *dpt* was measured via qPCR. There was no difference in expression between *mco4* knockdowns and wildtype (n= 3 replicates of 10 flies per genotype, t-test p>0.05).

day old adult male flies, there is no difference in microbial load between wildtype and *mco4* knockdown flies (Figure 7.4).
Figure 7.4 *mco4* Knockdowns Display Wildtype Early Adulthood Microbial Load

The relative levels of bacteria colonizing the gut of 2-day old adult flies was quantified by measuring the amount of bacterial DNA amplified via qPCR with 16S primers. There was no difference in microbial load between *mco4* knockdowns and wildtype flies (n=3 replicates of 10 flies per genotype, t-test p>0.05).
7.6 mco4 Knockdowns Display Small Deficiency in Survival that is Eliminated in Abiotic Conditions

To determine the effect of the PM on survival, flies were raised on normal laboratory food and axenic food treated with antibiotics. mco4 knockdown flies showed a slight but statistically significant decrease in viability when raised on normal food, but there was no difference in survival between wildtype and mco4 knockdown flies when raised on axenic food (Figure 7.5). The elimination of a survival defect of mco4 knockdown flies when raised on axenic food implicates a role of the PM for survival in the presence of bacteria.

7.7 Stressed One Week Old mco4 Knockdowns Display Increased Immune Activation and Slightly Lower Microbial Load

Previous studies have shown that the Drosophila gut microbiome can be reduced by daily transfers to fresh vials (Blum et al., 2013). To test if the PM may play a role in the establishment or maintenance of the microbiome, 2-day old male flies were placed in vials that were either transferred daily or never transferred for 7 days. There was a significant upregulation in immune activation as measured by dpt levels in mco4 knockdown flies that were transferred daily (Figure 7.6). In agreement with the literature, there was a decrease in microbial load in wildtype flies that were transferred daily compared to flies that were never transferred to fresh vials (Figure 7.7). mco4 knockdown flies also experienced a decrease in bacterial load for flies that were transferred
Figure 7.5 Antibiotic Food Eliminates Survival Defect in mco4 Knockdowns

Survival of flies was measured on normal laboratory food and antibiotic food. (A) mco4 knockdown flies died at a significantly more rapid rate than wildtype flies on normal food (n = 50 per genotype, median survival in days Da-Gal4 = 58, UASmco4RNAi = 57, UASmco4RNAi/Da-Gal4 = 50 days, Mantel-Cox Survival Test p<0.01). (B) mco4 knockdown flies survived at a wildtype rate when raised on antibiotics (n = 50 per genotype, median survival in days Da-Gal4 = 51.5, UASmco4RNAi = 48, UASmco4RNAi/Da-Gal4 = 49 days, Mantel-Cox Survival Test p = 0.4932).
Figure 7.6 Upregulation of Immune Activation in One-Week Old \textit{mco4} Knockdown Flies Transferred Daily

The relative levels of \textit{dpt} were measured in \textit{mco4} knockdown and wildtype flies transferred daily or not transferred at all over one week. \textit{mco4} knockdown flies transferred daily showed significant upregulation of \textit{dpt} compared to the other experimental groups (\(n=3\) replicates of 10 flies per group, ANOVA, Tukey post hoc \(p<0.01\)).
Figure 7.7 Decreased Bacterial Load in One-Week Old *mco4* Knockdown Flies

The relative levels of bacteria colonizing the gut of one-week old adult flies was quantified by measuring the amount of bacterial DNA amplified via qPCR with 16S primers. There was a decrease in load between wildtype flies transferred daily versus week old flies and a decrease in load in *mco4* knockdown flies compared to wildtype in both treatment groups respectively (n= 3 replicates of 10 flies per group, ANOVA, Tukey post hoc p<0.01).
daily compared to flies that were not transferred, but the knockdowns also displayed a much lower load than wildtype flies under similar conditions (Figure 7.7). These results suggest the PM may be necessary for microbiome maintenance or establishment.

**7.8 Lacking a PM Dramatically Alters Microbial Load and Induces a Profound **IMD**-Mediated Immune Response in 3-week Old Flies**

As flies age, their gut microbial load increases, their immune system becomes more activated and there is greater stem cell proliferation (Buchon et al., 2009). In the experiment with one-week old flies there was a small change in microbial load and no change in immune activation. The experiment of transferring flies daily was repeated but now for three weeks to drive gut instability and more accurately elucidate the role of the PM under more stressful conditions. After three weeks, there are striking changes in immune activation between flies with or without a PM, as mco4 knockdown flies show a significant increase in dpt expression compared to wildtype in both conditions (Figure 7.8). Inversely to dpt levels, mco4 knockdown flies show a significant decrease in microbial load compared to wildtype flies in both conditions (Figure 7.9).

While high levels of dpt indicate activation of the Lmd immune response pathway, to test if the Toll response pathway was activated we measured levels of drosomycin (drs), an antifungal peptide partially regulated by Lmd but primarily
Figure 7.8 Increased *imd* Mediated Immune Response in 3-Week Old *mco4* Knockdown Flies

The relative levels of dpt were measured in *mco4* knockdown and wildtype flies transferred to new vials daily or not transferred at all over 3 weeks. There was a significant increase in dpt expression in mco4 knockdown flies compared to corresponding treated wildtype flies (n=3 replicates of 10 flies per group, ANOVA, Tukey post hoc, p<0.01).
Figure 7.9 Decreased Microbial Load in 3-Week Old mco4 Knockdown Flies

The relative levels of bacteria colonizing the gut of 3-week old adult flies transferred to new vials daily or not transferred at all over 3 weeks was quantified by measuring the amount of bacterial DNA amplified via qPCR with 16S primers. There was a decrease in load in mco4 knockdown flies compared to wildtype in both treatment groups respectively (n= 3 replicates of 10 flies per group, ANOVA, Tukey post hoc p<0.01).
regulated by Toll (Sleiman et al., 2015). There was an observable increase in $drs$ expression between flies with and without a PM, however it was minimal in comparison to the change in expression of $dpt$ (Figure 7.10), suggesting the $Imd$ response pathway is the primary immune response activated in this experiment.

To determine if the differences in gut microbial load in the 3-week experiment altered microbiome composition, the gut microbiome of the four experimental groups was sequenced to determine if there were also changes in microbial diversity. The microbiome of laboratory raised $Drosophila$ has been characterized in several studies and typically is comprised of 8-12 families of bacteria normally defined by a dominant amount of acetobacter, followed by a significant load of lactobacillus (Fink et al., 2013, Staubach et al., 2013). Both wildtype and $mco4$ knockdown flies that were not transferred to fresh vials for 3 weeks display a microbiome pattern similar to those described in the literature (Figure 7.11, Table 7.1). Wildtype flies that were transferred to fresh vials daily show a microbiome with an overriding amount of acetobacter followed by several other families that measure less than 1% each of the total microbial load (Figure 7.11). $mco4$ knockdown flies that were transferred to fresh vials daily display a completely atypical microbiome, comprised of only 7.97% acetobacter and 1.69% lactobacillus, followed by several non-traditional microbiome families (Figure 7.11). The failure of flies lacking a PM to preserve a stable microbiome when stressed suggests the PM is necessary for microbiome maintenance or establishment.
Figure 7.10 Increased Toll Mediated Immune Response in 3-Week Old mco4 Knockdown Flies

The relative levels of drs were measured in mco4 knockdown and wildtype flies transferred to new vials daily or not transferred at all over 3 weeks. There was a significant increase in dpt expression in mco4 knockdown flies and the corresponding treated wildtype flies (n=3 replicates of 10 flies per group, ANOVA, Tukey post hoc, p<0.01).
Figure 7.11 Non-Traditional Microbiome Composition in 3-Week Old mco4 Knockdown Flies Transferred to New Vials Daily

The microbial composition of the mco4 knockdown and wildtype flies either transferred to new vials daily or not at all over 3 weeks was sequenced. Charts show families of bacteria that made up at least 1% of the total population. Both wildtype groups and the mco4 knockdown flies that were not transferred displayed a canonical laboratory raised microbiome dominated by acetobacter. mco4 knockdown flies transferred daily showed an atypical microbiome with 24 different families of bacteria making up at least 1% of the population.
Table 7.1 Breakdown of 3-Week Old Fly Microbiome Composition

7.9 Raising moc4 Knockdown Flies on Antibiotic Food Eliminates an Elevated IMD Immune Response

In the three-week vial transferring experiment there was a strong inverse relationship between bacterial load and Imd pathway activation, but it remained unclear if the elevated immune response was caused by invasive bacteria or if the reduction of the microbiome induces elevated immune activation as the microbiome has been shown to down-regulate innate immune responses (Kounatidis et al., 2012, Broderick et al., 2012). To answer this chicken or egg question, the three-week experiment was repeated but now with flies raised on axenic food. Every group of flies raised on axenic food showed lower dpt expression compared to similar treatment groups raised on normal food and no
significant difference between mco4 knockdown flies and wildtype (Figure 7.12). These results suggest the presence of bacteria causes an elevated immune response irrespective of the PM and the elevated dpt is dependent of bacteria.

**7.10 The presence of a PM is Partially Responsible for Changes in Microbial Load when the Microbiome is Stressed in an Immunocompromised Background**

The absence of bacteria eliminates the differences in immune responses in flies with or without a PM, but it remained unknown if the reduction of an immune response can alter gut microbial load differently in flies with or without a PM. To determine if the presence of a PM affects microbial load in immunocompromised flies, the 3-week transfer experiment was repeated on normal food but this time in an IMD mutant background. All four of the IMD mutant experimental groups showed around a 10-fold increase in microbial load compared to similar groups in a wildtype background, suggesting lacking an IMD immune response caused an increase in microbial load (Figure 7.13). However, there were significant differences in bacterial load between mco4 knockdown and wildtype flies in both the daily and no transfer groups, suggesting the presence of a PM plays a critical role in determining bacterial load (Figure 7.13). To assess the effect of lacking an IMD immune response on microbial diversity, the gut bacterial DNA of the four treatment groups in the IMD mutant background were sequenced.
Figure 7.12 Raising Flies on Axenic Food Eliminates Difference in Immune Response in 3 Week Experiment

The relative levels of dpt were measured in mco4 knockdown and wildtype flies raised on axenic food transferred to new vials daily or not transferred at all over 3 weeks. In contrast to flies raised on normal food, there was no difference in dpt levels among any of the experimental groups raised on axenic food (n=3 replicates of 10 flies per group, ANOVA p>0.05)
The relative levels of bacteria colonizing the gut of 3-week old adult flies transferred to new vials daily or not transferred at all over 3 weeks was quantified by measuring the amount of bacterial DNA amplified via qPCR with 16S primers. In an *imd* mutant background, *mco4* knockdown flies showed significantly higher bacterial loads in both daily transferred and non-transferred flies (*n* = 3 replicates of 10 flies, ANOVA, Tukey post hoc, *p*<0.01).
All four groups displayed a canonical microbiome dominated by *acetobacter* (Figure 7.14, Table 7.2). This result suggests the elimination of a typical microbiome in daily transferred *mco4* knockdowns in a wildtype *IMD* background is the result of the activation of the *IMD* pathway, as measured by an increase in *dpt* levels.

7.11 Upregulation of the JAK/STAT Stem Cell Renewal Pathway Only Observed in *mco4* Knockdown Daily Transferred Flies Raised on Normal Food

The cytokine *upd3* regulates the JAK/STAT pathway and its expression is induced upon damage to gut epithelial cells to promote wound healing and stem cell proliferation (Chakrabarti et al., 2016). The relative levels of *upd3* were measured in the 12 previously mentioned treatment groups (wildtype and *mco4* knockdown x daily or no transfer x normal or axenic food x wildtype or *Imd* mutant background) to assess gut homeostasis. The only group to display an increase in *upd3* expression was the daily transferred *mco4* knockdowns raised on normal food (Figure 7.15). Perhaps uncoincidentally, that treatment group was also the only group to display an atypical microbiome, suggesting the commensal microbiome regulates the JAK/STAT stem cell renewal pathway.
Figure 7.13  *imd* Mutant Background Does Not Eliminate Bacterial Load Difference in *mco4* Knockdowns in 3 Week Experiment

The relative levels of bacteria colonizing the gut of 3-week old adult flies transferred to new vials daily or not transferred at all over 3 weeks was quantified by measuring the amount of bacterial DNA amplified via qPCR with 16S primers. In an *imd* mutant background, *mco4* knockdown flies showed significantly higher bacterial loads in both daily transferred and non-transferred flies (*n* = 3 replicates of 10 flies, ANOVA, Tukey post hoc, *p*<0.01).
Figure 7.14 Traditional Laboratory Microbiome in imd Mutant Background

The microbial composition of the mco4 knockdown and wildtype flies in an imd mutant background either transferred to new vials daily or not at all over 3 weeks was sequenced. Charts show families of bacteria that made up at least 1% of the total population. All experimental groups display a canonical laboratory microbiome with a dominant population of *acetobacter*. 
Table 7.2 Breakdown of 3-Week Old Fly Microbiome Composition in *imd*
Mutants

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<th>mco4 Knockdown No Transfer</th>
<th>mco4 Knockdown Daily Transfer</th>
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Figure 7.15 Upregulation of *upd3* in *mco4* Knockdowns Transferred Daily on Normal Food

The relative level of *upd3* was measured via qPCR in all the experimental groups for the three 3-week long experiments. *upd3* was upregulated only in *mco4* knockdown flies transferred daily on normal food with a wildtype *imd* (N=3 replicates of 10 flies per group, ANOVA, Tukey post hoc, p<0.01).
7.12 Discussion

Experiments here suggest an integral role of the PM for maintaining stable host-microbiome interactions and gut homeostasis. The PM does not influence gut pH compartmentalization, defecation rate or early adulthood microbial load and immune response, but greatly impacts the microbial community and immune response as flies age and when the microbiome is stressed.

It remains unclear why *drd* mutants and *cyp6t1* and *cyp6a22* knockdown adults display reduced defecation (Chapter 5) while *mco4* knockdown flies do not. It is possible *drd, cyp6t1* and *cyp6a22* play a role in non-PM related gut physiological development that is necessary for normal defecation as adults. It is also possible *drd, cyp6t1* and *cyp6a22* are necessary for the formation of an additional layer of the PM, or perhaps the mucosal layer, that *mco4* is not, and that layer is necessary for normal defecation. Further study will be needed to understand the mechanism for reduced defecation in *drd* mutants and *cyp6t1* and *cyp6a22* knockdowns. Additionally, *mco4* knockdown flies do not show an upregulation in early adulthood immune activation, as measured by 2-day old *dpt* levels, while *drd* mutants and *cyp6t1* and *cyp6a22* knockdowns do (Chapter 5). When combined with the similar pattern of observations of defecation rates, this result provides more evidence to the observation that the guts of *drd* mutants and *cyp6t1* and *cyp6a22* knockdowns are different from *mco4* knockdowns.

The nearly wildtype lifespan of *mco4* knockdowns suggests the PM is not incredibly important for survival in a laboratory setting and it seems unlikely *mco4*
knockdown flies are suffering from acute starvation. However, the observation
that raising flies axenically can eliminate the survivorship difference in wildtype
and *mco4* knockdown flies suggests the presence of bacteria explains the
difference between wildtype and *mco4* flies under normal conditions. Perhaps
*mco4* knockdown flies are more susceptible to infection or, as results from this
chapter suggest, *mco4* knockdowns experience an increased immune activation
and stem cell renewal upregulation as they age, which has been shown to lead to
gut dysbiosis and earlier lethality in a previous study (Broderick et al., 2014).

Interestingly, changes in microbial load and immune activation are much
more pronounced in *mco4* knockdowns the older the flies are and the longer the
microbiome is stressed with daily vial transfers. These observations suggest a
role of the PM in microbiome maintenance or establishment. It has been reported
that gut bacterial load increases with age (Wong et al., 2016), therefore, flies
lacking a PM appear to accumulate bacteria at a slower rate than wildtype flies.
It remains unknown if commensal bacteria physically attaches to the PM and the
removal of the structure is the cause of the lower load. It is also noteworthy that
under normal laboratory conditions immune activation and microbial load are
inversely related, in both wildtype and *mco4* knockdowns, prompting a chicken or
egg question – does the more abundant, stable microbiome suppress immune
response or does the elevated immune response ablate the microbiome?
Experiments under axenic conditions show no elevated immune response in
wildtype or *mco4* knockdowns, suggesting the presence of the PM in the
absence of bacteria has no effect on immune response. In an
immunocompromised background there is an increase in bacterial load in all experimental groups compared to wildtype immune backgrounds, suggesting the immune response pathway is at least partially responsible for lowering bacterial load. The elevated $Imd$ pathway activation is very impactful in 3-week daily transferred $mco4$ knockdowns as these flies appear to lack a microbiome based on abundance and microbial diversity, both of which can be at least partially rescued in an $Imd$ mutant background. Wildtype flies transferred daily for 3 weeks still maintained stable microbial diversity, suggesting the PM is necessary for maintaining a stable microbial community when the microbiome is stressed. Additionally, in an $imd$ mutant background, both wildtype $mco4$ treatment groups and the $mco4$ knockdown with no transfer treatment group harbored a higher percentage of “non-traditional” laboratory microbiome members compared to the same treatments with wildtype $imd$ (Table 7.1, Table 7.2). This result suggests that loss of an $imd$-mediated immune response allowed for broader microbial colonization of the gut. The observation of a more heterogeneous microbiome population in the absence of a functional $imd$ agrees with a previous study that showed changes in expression of immune response factors modulated by $caudal$ altered microbiome composition (Ryu et al., 2008) and suggests a functional immune system is necessary to maintain a “traditional” laboratory microbiome.

Intriguingly, in a wildtype immune background, both wildtype and $mco4$ knockdown flies lose their $Lactobacillus$ population when transferred to new vials daily. $Lactobacillus$ family members have a generation time of 66-87 mins while $Acetobacter$ generation times can be as low as 20 minutes (Altermann 2005), so
perhaps *Lactobacillus* populations need longer to establish, and the daily transfers do not allow enough time for *Lactobacillus* to gain a foothold in the presence of an immune response.

The striking elevation of the JAK/STAT pathway measured by *upd3* expression in only the 3-week old daily transferred *mco4* knockdowns underscores the integral role of the PM in maintaining gut homeostasis. This was also the only experimental condition that displayed a non-canonical microbiome diversity, perhaps not even a microbiome at all considering the extremely low level of bacterial abundance. The microbiome is known to downregulate JAK/STAT signaling (Broderick et al., 2014) and when flies lack a PM and their microbiome is stressed via daily transfers, the flies lose their microbiome, and experience an upregulation in the stem cell renewal pathway.

A caveat of the results in this chapter is the varying levels of *Wolbachia* found in each bacterial sample. *Wolbachia* is not a member of the commensal microbiome and is transferred generationally (Sannino 2018) and its presence may influence the total bacterial load calculations presented here. The microbial diversity data presented removed *Wolbachia* to focus on gut populations, but in the raw sequencing data there was a difference in total *Wolbachia* between samples. Since the differences in total bacterial load between groups were so vast, 10-100 fold between groups of interest, the varying levels of *Wolbachia* in each sample likely will not alter the overall comparisons reported here. However, the exact changes in gut bacterial loads between groups could be more precisely calculated in future experiments in flies without *Wolbachia*. 
The results from this chapter underscore the importance of the PM in maintaining adult fly gut homeostasis. Flies lacking a PM tend to more readily lose their commensal microbiome, experience an increase in *Imd* immune activation and experience an upregulation of gut stem cell renewal (Figure 7.16). As interest in studying microbiome-host relationships continues to increase, it will be valuable to continue to investigate the role barrier structures play in modulating these relationships.

![Figure 7.16 Model of Gut Dynamics Influenced by PM when Microbiome is Stressed](image)

When the microbiome is stressed by daily transfers to new vials, flies with a PM can maintain gut homeostasis while flies lacking a PM experience changes in immune activation, bacterial load and composition and stem cell upregulation.
Chapter 8: Conclusion

Maintenance of functional barrier structures separating an organism from and regulating its interaction with the environment is critical for many aspects of fitness. Arguably, the most dynamic interface between an animal and its environment is the gut, as the organism must balance the primary gut functions, digestion and nutrient acquisition, with several homeostatic and defensive systems including pathogen defense, microbiome maintenance and stem cell renewal. Many animals secrete extracellular barrier structures along their guts to organize and modulate these interactions. As with many insects, *Drosophila* excrete a semi-permeable structure along its midgut called the peritrophic matrix (PM), which is theorized to function in numerous gut systems. However, to date, all studies of PM function are limited as there are no published insect lines that completely lack a PM and most studies utilize “leaky” PM models with a partially impaired PM. In this work, I report four genes necessary for PM synthesis: *drd*, *mco4*, *cyp6a22* and *cyp6t*. Using knockdowns of these genes, I investigated the genetic requirements for PM formation and characterized the role of the PM in several gut systems.

8.1 *drop-dead* Mutants Lack a PM

Our lab recently observed a novel phenotype of *drd* mutants, the absence of a PM, the first reported insect line to completely lack a PM. *drd* mutants display a diverse range of phenotypes across numerous tissues including early adult lethality, female sterility and gut dysfunction. In this work, I showed
constant robust expression of \textit{drd} in the adult cardia is necessary for PM synthesis. Through temporal and spatial knockdown and rescue of \textit{drd}, I separated the lack of PM phenotype from the early adult lethality and the gut dysfunction phenotype of reduced adult defecation. Previous work in our lab showed \textit{drd} expression during metamorphosis is necessary and sufficient for adult survival, suggesting \textit{drd} plays a critical developmental role, but adult expression of \textit{drd} is not necessary for adult survival (Sansone and Blumenthal 2013). Here, experiments assaying for PM presence utilizing adult knockdown and rescue of \textit{drd} show constant adult expression of \textit{drd} is necessary for PM formation, suggesting \textit{drd} is necessary during adulthood for at least one physiological process, and that the \textit{drd} gene product is likely functioning throughout adulthood in the PM synthesis pathway.

The diverse phenotypes throughout various tissues of \textit{drd} mutants has fascinated our lab for years, and the observation that \textit{drd} appears to be necessary for a specific temporal developmental process in one tissue and a maintenance process at a different life stage in another tissue adds to the intrigue to further investigate \textit{drd}. Currently, the biochemical function of \textit{drd} remains unknown, and elucidating this mechanism in future studies could enlighten why and how \textit{drd} is necessary for such diverse processes, at different stages in development, in various tissues. Sequence homology to bacterial acyl-transferases predicts DRD as an acyl-transferase, however this hypothesis remains untested. Based on enhancer screens and a study of family homologues in worms, \textit{drd} may be involved in lipid processing (Watts 2006), but
this also remains untested, and is an incredibly broad statement, as lipid modification is a signaling mechanism for numerous processes throughout cells and tissues. Elucidating drd biochemical function in future work could be valuable to understanding the nature of multifunctioning genes.

While drd mutants could be a valuable tool to study PM function as the only reported insect line to fully lack the structure, the diverse phenotypes associated with drd mutants may complicate interpreting experimental results. The previously mentioned ability to separate the gut phenotypes of lacking a PM and reduced defecation would suggest lacking a PM is not causal to reduced defecation, and that drd is potentially functioning in another gut process. This knowledge would temper confidence in claiming solely the PM as the factor influencing potential experiments examining PM function in the gut with drd mutants or knockdowns. To identify additional genes necessary for PM synthesis that may produce less pleiotropic models as well as identify genes that may be functioning in similar pathways as drd to potentially shed light on drd function, I performed a series of RNAi screens for the presence of a PM. These screens revealed expression of cyp6a22, cyp6t1 and mco4 in the cardia as necessary for PM synthesis.

8.2 cyp6a22 and cyp6t1 are Necessary for PM Synthesis

Identification of cyp6a22 and cyp6t1 as necessary for PM synthesis was exciting not only because they could serve as potential models for PM physiological function, but also due to the possibility that they may be functioning
in a similar pathway as \textit{drd}. These two cytochrome p450s were candidate genes in an RNAi screen because they displayed significantly altered expression in a microarray conducted in stage 10 egg chambers of \textit{drd} knockdown flies (Blumenthal unpublished). It was curious that genes with altered expression in a \textit{drd} knockdown background in one tissue seem to phenocopy \textit{drd} mutants in another tissue but may hint a global biochemical function of \textit{drd} versus a tissue specific function. \textit{Drosophila} have 90 identified cytochrome p450 genes and are typically associated with either hormone signaling or defense due to their ability to metabolize endogenous and xenotropic substrates (Estabrook 2003, Good 2014). \textit{cyp6a22} and \textit{cyp6t1} are expressed throughout the fly but their function was unknown (flyatlas.com). In a series of experiments, I showed global \textit{cyp6a22} and \textit{cyp6t1} knockdowns phenocopied \textit{drd} mutant gut phenotypes of lacking a PM, reduced defecation and early adulthood immune activation, however, unlike \textit{drd} mutants, \textit{cyp6a22} and \textit{cyp6t1} knockdown females were fertile and did not suffer from early adult lethality but rather displayed wildtype 40-day survival rates. These results hint that \textit{cyp6a22} and \textit{cyp6t1} are necessary in similar gut processes in which \textit{drd} is also necessary, but, unlike \textit{drd}, \textit{cyp6a22} and \textit{cyp6t1} are not necessary for female fertility or adult survival. I cannot rule out that \textit{cyp6a22} and \textit{cyp6t1} are not in the same female fertility or adult survival pathway as \textit{drd}, just that they are not necessary. Additionally, I can not be certain \textit{drd}, \textit{cyp6a22} or \textit{cyp6t1} are even in the same PM biochemical synthesis pathway, as \textit{drd} mutants may lack a PM by a different mechanism than \textit{cyp6a22} or \textit{cyp6t1} knockdowns. This work primarily characterized the various phenotypes
of cyp6a22 and cyp6t1 knockdowns, and future studies will need to elucidate if either of these cytochrome p450s are interacting with drd directly or in similar pathways anywhere in the fly. A major unanswered question about drd is whether it is performing similar roles in different tissues or if drd functions uniquely in various processes. Understanding the relationships between drd and cyp6a22 and cyp6t1 throughout the fly could shed light on this mystery.

8.3 mco4 is Necessary for PM Formation

Perhaps the most significant and fruitful scientific contribution of this dissertation was the identification of mco4 expression in the cardia as necessary for larval and adult PM synthesis in Drosophila. RNAi knockdown of mco4 either globally or in a gut specific manner reliably produced flies that completely lacked a PM, and unlike drd mutants or cyp6a22 and cyp6t1 knockdowns, mco4 knockdowns did not display non-PM related phenotypes, making them the ideal model to study PM physiological function (Table 8.1). mco4 is expressed at high levels and nearly exclusively in the cardia, and collaborative work with Neal Dittmer, PhD, Research Professor at Kansas State University, showed MCO4 to behave biochemically as a laccase, a well conserved family of enzymes implicated in extracellular barrier synthesis and cuticular formation (Kudanga et al., 2017, Forootanfar et al., 2015). Arguably, mco4 knockdown flies are the best available model to study PM physiological function, far superior to “leaky” models due to the complete lack of the structure and superior to drd, cyp6a22 and cyp6t1
Table 8.1 Description of Phenotypes in Knockdown of Genes Necessary for Adult PM

<table>
<thead>
<tr>
<th>Phenotypes</th>
<th>drd</th>
<th>mco4</th>
<th>cyp6t1</th>
<th>cyp6a22</th>
</tr>
</thead>
<tbody>
<tr>
<td>Larval PM</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Adult PM</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Reduced Adult Defecation</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Acute Early Adult Lethality</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

models due to less pleiotropy, and therefore can be used to examine PM function in depths previously unavailable.

The evolutionary history of the mco family of genes in insects suggests the Drosophila mco4 may have acquired its current role in PM synthesis from a more ancient laccase. Of the insect genomes sequenced and annotated, all species contain at least 2 members of the mco family, which appear to have homologous sequences and function, however, some Dipteran species have additional and unique mco family members (Peng et al., 2015). For example, Drosophila melanogaster have 4 mco family members while the mosquito Anopheles gambiae genome contains 5 members (Lang et al., 2012). mco1 and mco2, share sequence homology in flies and mosquitos, but the other mco genes lack homology and therefore likely evolved independently (Gorman et al., 2008). If mco4 is necessary for PM synthesis in Drosophila but it does not have orthologs in the genome of insects that also have PMs, it is possible mco4 took over the role of PM synthesis in Drosophila from a more general laccase. Elucidating if
and what other mco genes are necessary for PM synthesis in other insects could shed light on the evolutionary history of laccases.

8.4 Construction of mco4 Genetic Tools

In stark contrast to the great fortune of uncovering the power of mco4 knockdowns in an RNAi screen, the most disappointing series of results reported here was the inability to create additional mco4 based genetic tools. While nearly any experiment investigating PM function could potentially be conducted using mco4 knockdown flies, an mco4 mutant line would allow for more rapid investigation and could validate results observed in knockdowns. Since RNAi knockdown of mco4 using a variety of global or gut specific drivers readily produced large quantities of flies lacking a PM coupled with the nearly wildtype length of adult survival in mco4 knockdown flies, it was assumed an mco4 mutant line would be healthy and easy to maintain. I created a mco4 mutant with CRISPR, however, maintaining the line was difficult. Even when the mutated mco4 chromosome was placed over a balancer chromosome, the line yielded very few mutant offspring, most of which would die within a week of eclosion, reducing the potential efficacy of the mco4 mutant line for PM physiological experimentation. The one silver lining from the creation of the mco4 mutant line was that the mutant flies did lack a PM, further suggesting mco4 is necessary for PM synthesis. It remains mysterious why the mco4 knockdowns and mutants display such divergent survival phenotypes, but a possible explanation is that mco4 is involved in some unknown early embryonic development process and
RNAi induced via the GAL4-UAS system knocks \textit{mco4} down after this critical timepoint. Future experiments investigating when and how \textit{mco4} mutants die could potentially shed light on why knockdowns and mutants display such different survival rates.

While there are hundreds of GAL4 lines that express in the \textit{Drosophila} gut, there is no published line that exclusively drives expression in the cardia. Since the endogenous expression of \textit{mco4} is confined to the cardia, it was theorized an \textit{mco4}-GAL4 line could be the first line to drive expression solely in the cardia. An \textit{mco4}-Gal4 line could potentially be crossed with \textit{drd} RNAi to tease apart the \textit{drd} PM and non-PM gut phenotypes. However, despite inserting the \textit{mco4} promoter sequence into four different GAL4 backbone vectors with varying levels of GAL4 expression and multiple rounds of injections of these plasmids into embryos conducted by companies specializing in this technique, I was unable to generate an \textit{mco4}-GAL4 line. Perhaps if \textit{mco4} does play a vital role in early embryonic development, as potentially hinted by the health of \textit{mco4} mutant line, and is expressed at high levels, the production of GAL4 protein in this pattern may be lethal to the embryo, hence no viable flies. Why an abundance of GAL4 protein may cause embryonic lethality is difficult to speculate, but it is also difficult to explain why an \textit{mco4}-GAL4 line could not be created using commonly used techniques.

An \textit{mco4}-UAS line could enable \textit{mco4} temporal expression experiments designed to more accurately define the genetic requirements for PM synthesis. Unfortunately, multiple rounds of injections of a plasmid containing the \textit{mco4} cds
failed to yield any transformed flies. Again, the inability to generate an *mco4* based genetic tool is both frustrating and challenging to rationalize. Our lab has used the same injection company and same plasmid backbone to successfully create UAS lines in the past, so it is mysterious why an *mco4*-UAS could not be created. Perhaps an additional copy of the *mco4* cds inside the cell is toxic, which admittedly seems unlikely, but it is difficult to explain this result.

### 8.5 *mco4* Knockdown Larva Display Mild Starvation due to Digestive Inefficiency

The PM has been theorized to play an integral role in digestion and this work utilized *mco4* knockdown larvae to examine the extent and mechanism by which the *Drosophila* larval PM promotes digestive efficiency. *mco4* knockdown larvae developed and gained weight more slowly than wildtype, and *dilp* expression patterns from knockdown larvae indicated these developmental defects were due to starvation. Food movement through the gut was unaffected by the absence of the PM but when raised on a more nutritious food source, *mco4* knockdown larva experienced a significant rescue in body mass, suggesting the PM plays a role in digestion but not in the movement of food through the gut. The mechanism by which the PM enhances digestion remains unknown, as it is technically difficult to tease apart different aspects of digestion, such as separating digestive enzyme efficiency from nutrient absorption in a reliable manner. In the future, perhaps as new techniques become available,
additional experiments will be needed to elucidate the mechanism by which the
*Drosophila* PM enhances digestion.

While it is unsurprising the *Drosophila* PM is necessary for maximum
digestive efficiency, the phenotypes observed in *mco4* knockdown larvae are
mild compared to other insect species with “leaky” PM models. For example, a
study investigating the physiological consequence of disrupting the larval PM
structure via knockdown of PM components in the potato beetle, *Leptinotarsa
decemlineata*, showed adults with “leaky” PMs as larvae only survived pupation
about 20% of the time and those that survived to adulthood were roughly 40%
lighter than wildtype (Shi et al., 2016), both much more extreme from the results
reported in this work. One explanation for these differences may be the
adaptiveness of laboratory *Drosophila* to standard laboratory food. Flies
obtained for the experiments in this work came from large stock centers, and
may be derived from lines raised on similar food sources and conditions for
dozens to hundreds of generations. Conversely many non-*Drosophila* insect
studies create their own stocks from the wild or use stocks that may only have
been bred in the lab for only a few generations (Osman et al., 2015). Therefore,
it must be considered that stock center based *Drosophila* have had more time to
adapt to and may have become more proficient at digesting laboratory food.
Disruptions to the PM may not appear to affect *Drosophila* as much as other
insects simply because they are better adapted to processing their food source,
and additional experiments examining *Drosophila* PM digestive functionality on
unique food sources will be necessary to determine if this is a contributing factor to results reported here.

Another explanation for why disruption of the Drosophila PM may not yield as extreme results of digestive inefficiency as other insects is that the PM in other species is necessary to produce a countercurrent flow of food and digestive enzymes. Relative to other insects used to study PM function such as Spodoptera frugiperda or Erinnyis ello, Drosophila have a physically long midgut (Hegedus 2009). Therefore, food stays in the midgut longer as it travels through the digestive tract and has more time to interact with digestive enzymes. Many insects with shorter midguts create a countercurrent flow of enzymes and partially digested food in the ectoperitrophic space, through the pumping of water in the posterior of their midgut, which theoretically facilitates more dynamic and numerous interactions (Caldeira et al., 2007). It is possible the PM is necessary for facilitation of this countercurrent flow in non-Drosophila insects, and therefore loss or disruption of the PM in these species could have a greater impact on digestion. Since midgut morphology and digestive processes vary so greatly among insects, it would be useful to identify models of insects with shorter midguts utilizing countercurrent flow that completely lack a PM in the future to better understand differences of PM digestive function in diverse insects.

8.6 PM is Necessary for Maintaining Adult Gut Homeostasis

Drosophila has recently emerged as a leading model to study host-microbiome interactions, however, no published studies investigate how the PM
may modulate these interactions. Work presented here suggests a critical role of the PM in promoting homeostasis between the microbiome, immune system and stem cell renewal pathways. \textit{mco4} knockdown flies survive at a slightly less than wildtype rate on normal food, but this difference is eliminated when raised on antibiotic food, highlighting a relationship between the PM and bacteria. Lacking a PM as 2-day old and one-week old flies does not greatly alter immune activation or microbial load, but at three weeks of age \textit{mco4} knockdown flies exhibited striking differences in bacterial load and immune activity. Stressing the microbiome with daily vial transfers essentially eliminated the microbiome in three-week old \textit{mco4} knockdown flies but not wildtype flies, suggesting the PM is necessary for microbiome maintenance. Experiments with antibiotic food showed hyper induction of the immune system in \textit{mco4} knockdown flies is due to the presence of bacteria and not the lack of the PM itself. Experiments with \textit{imd} mutants showed activation of the immune system alone is not driving changes in microbial load in \textit{mco4} knockdowns but activation of the \textit{imd} pathway is at least partially responsible for the loss of a microbiome in flies with a stressed microbiome. Finally, significant upregulation of stem cell renewal was only observed in \textit{mco4} knockdown flies that also lacked a microbiome, highlighting the importance of the PM in maintaining overall gut homeostasis.

Upregulation of \textit{upd3} in \textit{mco4} knockdowns with a stressed microbiome indicate a wound healing response, but the nature of assumed damage sustained by gut epithelial cells remains unknown. It is possible physical abrasions by food or microbes that would be confined to the endoperitrophic
space under wildtype conditions may induce cellular damage in flies lacking a PM. Alternatively, if ROS release into the gut lumen is increased in flies lacking a PM, an untested theory in this work, these factors may cause cellular damage. Another possibility is that there is no actual damage to the cells, and the upregulation of *upd3* is a consequence of lacking a microbiome, as a commensal bacterial population has been shown to down regulate activation of the JAK/STAT pathway (Buchon et al., 2009). Teasing apart the cause and effect nature of observations seen in flies lacking a PM will be critical in understanding gut defense and homeostatic functions.

Results reported here demonstrated how the PM impacts the dynamic relationships between the *imd* pathway, microbiome and *JAK/STAT* pathway, but there are likely additional gut defensive and homeostatic functions also modulated by the PM that remain underexplored. *Duox* is a plasma membrane-expressing NADH oxidase and releases ROS into the gut lumen in the presence of bacteria as a defensive measure (Ha et al., 2005). Since findings here support a critical role of the PM in immune function, it is possible the PM may partially regulate ROS release via *duox*, and future experiments will be needed to investigate this relationship. Also unexplored in this work is the role of the larval PM on larval microbiome dynamics. In adult *mco4* knockdowns the microbial population is highly altered compared to wildtype and *mco4* knockdown larva display a mild starvation phenotype. It is possible lacking a larval PM alters the larval microbiome in *mco4* knockdowns and future studies investigating this hypothesis could shed light on the mechanism for the digestive inefficiency
observed in *mco4* knockdown larvae. Additionally, use of *mco4* knockdown flies subjected to pathogenic infection can enhance understanding of the defensive functionality of the PM. “Leaky” PM *Drosophila* models show an increased susceptibility to pathogens, suggesting the PM is necessary for an optimal response to pathogenic invasion (Hori et al., 2018), and future studies utilizing *mco4* knockdown flies can more accurately describe the role of the PM in pathogenic defense.

### 8.7 Unanswered Questions and Potential Implications from this Work

Another caveat from claims in this work is the definition of “lacking a PM”. Reported here are four genes that apparently lack a PM based on gross dissection of the midgut as well as chitin staining. It remains unknown if less noticeable, non-chitin layers of the PM still exist in these knockdowns, and future studies investigating the structure of these guts will be needed to more accurately define the nature of this “lack of a PM”. It is also unknown if other, non-PM barriers, such as the mucosal layer, are affected by these knockdowns.

As interest in understanding host-microbiome relationships continues to become more popular, application of *Drosophila mco4* knockdown flies as a model for understanding gut barrier-microbe interactions may prove very fruitful. A recent study utilizing mouse models investigated the relationship among gut mucosal layers, microbiome composition and organismal fitness and showed disruption to the extracellular gut lining altered microbial load and led to developmental defects (Schroeder et al., 2018). These findings in mice are
similar to results reported here in *Drosophila* and underscore the power of using the inexpensive and easily experimentally manipulated *Drosophila mco4* knockdowns as a model to study gut barrier-microbiome interactions. Understanding how microbes interact with extracellular gut barriers could enhance development and efficacy human probiotic and anti-inflammatory treatments. The lactic acid bacterium *Lactococcus lactis* is found in many dairy products and is ingested in large quantities by humans across the globe (Rintahaka et al., 2015). *L. lactis* has been shown to exhibit mucosal binding activity, and has been considered as a vehicle for delivering in vivo therapeutic molecules to the human intestine (Mercier-Bonin et al., 2017). Future experiments utilizing *Drosophila mco4* knockdowns can potentially shed light on how extracellular structural binding bacteria interact with their host and can serve as a model laying the groundwork for human gut medical technologies.

In addition to human medical relevance, this work may contribute to managing agricultural pest control and vector borne illness spread by insects. Inefficient agricultural yields threaten the global food supply, and damage to crops by pests can lower total output by 30-50% (Cerda et al., 2017). Another major global concern is the spread of vector diseases by mosquitoes, which account for over a million deaths a year (Caraballo 2014). Genetic disruption or complete ablation of the PM in crop pest insects and mosquitoes may reduce the amount of crops lost and lower the spread of disease. Results here show a decrease in survival rate in flies lacking a PM as well as digestive inefficiencies. Reducing the number of pests and mosquitoes or decreasing the ability to
consume or spread disease could prove a viable strategy in crop and disease management. Recognizing genetic targets that may damage or remove a PM in major crop pests and mosquitos could be an effective form of control, and the identification of *drd, cyp6t1, cyp6a22* and *mco4* as necessary for PM synthesis in *Drosophila* can inform candidate genes to test for PM synthesis in other insect species. The ecological impact of insects without PMs would need to be considered, but genetic disruption of the PM may be a valuable tool in the fight against crop loss and disease.

This work offers novel insights into the genetic requirements for PM synthesis in *Drosophila* and uses the first reported insects completely lacking a PM to characterize the role of the PM within several systems in depths previously unexplored. These studies provide significant contributions to the fields of insect genetics and physiology and provide powerful models for future studies to investigate the influence of gut barrier structures in host-environment interactions.
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