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# Pleiotropic and novel phenotypes in the *Drosophila* gut caused by mutation of *drop-dead*

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ABSTRACT

Normal gut function is vital for animal survival, and deviations from such function can contribute to malnutrition, inflammation, increased susceptibility to pathogens, diabetes, neurodegenerative diseases, and cancer. In the fruit fly *Drosophila melanogaster,* mutation of the gene *drop-dead (drd)* results in defective gut function, as measured by enlargement of the crop and reduced food movement through the gut, and *drd* mutation also causes the unrelated phenotypes of neurodegeneration, early adult lethality and female sterility. In the current work, adult *drd* mutant flies are also shown to lack the peritrophic matrix (PM), an extracellular barrier that lines the lumen of the midgut and is found in many insects including flies, mosquitos and termites. The use of a *drd-gal4* construct to drive a GFP reporter in late pupae and adults revealed *drd* expression in the anterior cardia, which is the site of PM synthesis in *Drosophila*. Moreover, the ability of *drd* knockdown or rescue with several gal4 drivers to recapitulate or rescue the gut phenotypes (lack of a PM, reduced defecation, and reduced adult survival 10–40 days post-eclosion) was correlated to the level of expression of each driver in the anterior cardia. Surprisingly, however, knocking down *drd* expression only in adult flies, which has previously been shown not to affect survival, eliminated the PM without reducing defecation rate. These results demonstrate that *drd* mutant flies have a novel phenotype, the absence of a PM, which is functionally separable from the previously described gut dysfunction observed in these flies. As the first mutant *Drosophila* strain reported to lack a PM, *drd* mutants will be a useful tool for studying the synthesis of this structure.

## 1. Introduction

Obtaining energy from food is vital for animal survival, and deviations from normal digestive function can have significant impacts on organismal fitness. The gut of *Drosophila melanogaster* is a well characterized model for studying digestive function (Guo et al., 2016; Lemaitre and Miguel-Aliaga, 2013). In adult flies, ingested food is stored in the crop. For digestion to occur, the crop contracts and food passes through the stomodeal valve of the cardia into the midgut (Gelperin, 1971; Stoffolano and Haselton, 2013). As in many other insect species, the midgut of both larval and adult *Drosophila* is lined with the PM, a semipermeable extracellular barrier (Hegedus et al., 2008; Lehane, 1997; Terra, 2001). Nutrients pass through the PM and are absorbed by the gut epithelial enterocytes (Lemaitre and Miguel-Aliaga, 2013). Chemical or enzymatic ablation of the PM in the larvae of multiple insect species has demonstrated a role for this structure in digestive efficiency and protection against ingested pathogens (Barbehenn, 2001; Bolognesi et al., 2001; Rao et al., 2004; P. Wang and Granados, 2000).

Mutation of the *Drosophila* gene *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, while still capable of contracting, 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 become depleted in the mutants. Therefore, it appears that *drd* mutant flies exhibit a starvation phenotype despite ingesting food (Peller et al., 2009).

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We initially 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 stomodeal valve in the cardia (Peller et al., 2009). However, in subsequent work we separated these two phenotypes 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 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.

Here, we continue our characterization of *drd* mutants and report a novel and unique gut phenotype of *drd* mutants: lack of a PM. We find that continuous expression of *drd* in the adult is necessary for PM formation, but surprisingly, lacking a PM is not the sole cause of *drd* mutant gut dysfunction nor does the absence of a PM appear to contribute to early adult lethality.

## 2. Materials and methods

### 2.1. 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 12 h:12 h 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 $drd^{GD15915}$ *UAS-Dcr-2* and *UAS-Dcr-2* $drd^{GD3367}$ lines were created previously by recombination between VDRC stocks $w^{1118}$*;P{GD3367}v37404* (FBst0461992) and $w^{1118}$*; P{GD15915}v51184* (FBst0469325) and Bloomington stock $w^{1118}$*; P{UAS-Dcr-2.D}2* (FBst0024650, RRID:BDSC_24650) (Sansone and Blumenthal, 2012). Other stocks ($w^{1118}$*; P{UAS-GFP.nls}14* (FBst0004775, RRID:BDSC_4775), $w^{*}$*; P{GAL4-Hsp70.PB}2* (FBst0002077, RRID:BDSC_2077), $w^{*}$*; P{UAS-lacZ.B}Bg4-2-4b* (FBst0001777, RRID:BDSC_1777), $w^{1118}$*; P{GawB}DJ717* (FBst0008180, RRID:BDSC_8180), $w^{1118}$*; P{GawB}DJ626* (FBst0008166, RRID:BDSC_8166), $w^{1118}$*; P{GawB}DJ628* (FBst0008167, RRID:BDSC_8167), $y^{1}$ $w^{*}$ *Mi{*$y^{+mDint2}$*=MIC}*$drd^{MI15121}$*/FM7h* (FBst0059743, RRID:BDSC_59743), $y^{1}$ $w^{*}$*; P{*$w^{+mC}$*=loxP(Trojan-GAL4.1)}12B; *$Dr^{1}$*/TM3, Sb*$^{1}$ (FBst0060304, RRID:BDSC_60304), $y^{1}$ $w^{67c23}$*; sna*$^{Sco}$*/CyO, P{*$w^{+mC}$ *= Crew}DH1* (FBst0001092, RRID:BDSC_1092), $y^{1}$ *M{3xP3-RFP.attP}ZH-2A* $w^{*}$*; M{vas-int.Dm}ZH-102D* (FBst0024480, RRID:BDSC_24480), and *FM7i/C(1)DX,* $y^{1}$ $f^{1}$ (FBst0005263, RRID:BDSC_5263)) were obtained from the Bloomington *Drosophila* Stock Center. The line bearing the second chromosome *UAS-drd* on a $drd^{lwf}$ background was described previously (Sansone and Blumenthal, 2012). The genes and alleles referenced in this work include *drd* (FBgn0260006), $drd^{lwf}$ (FBal0193421), $drd^{1}$ (FBal0003113), and $drd^{MI15121}$ (FBal0302603). Stocks were not outcrossed prior to this study.

For rescue experiments with two copies of the *DJ717-gal4* driver, the *UAS-drd* 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-drd* 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 $drd^{lwf}$ background.

### 2.2. 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 h-overnight. Midguts were then dissected in PBS, fixed in 4% PFA/PBS for 30 min, washed 3 × 10 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 min. The guts were then fixed, washed, and mounted as above.

### 2.3. Gut histology

Flies were decapitated and the posterior tip of the abdomen removed and were then fixed in 2% glutaraldehyde, buffered at pH 7.3 with 50 mM Na-cacodylate and 150 mM saccharose. Postfixation was carried out in 2% osmium tetroxide in the same buffer. After dehydration in a graded acetone series, tissues were embedded in Araldite and sectioned with a Leica EM UC6 ultramicrotome. Semithin 1 µm sections were stained with methylene blue and thionin and viewed with an Olympus BX-51 microscope.

### 2.4. X-gal staining

X-gal staining in adults was performed as previously described (Choi et al., 2008). The digestive system was dissected in PBS on ice, fixed in 1% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) in PBS for 10 min, and washed in 1x PBS for 1 h at room temperature. The digestive system was stained with 0.2% X-gal (IBI Scientific, Peosta, IA) in staining buffer (6.1 mM $K_4Fe(CN)_6$, 6.1 mM $K_3Fe(CN)_6$, 1 mM $MgCl_2$, 150 mM NaCl, 10 mM $Na_2HPO_4$,10 mM $NaH_2PO_4$) in the dark at room temperature for 30 min (*DJ717-gal4*, *DJ626-gal4*) or 1 h (*DJ628-gal4*). The digestive system was then mounted in VectaShield Mounting Medium with DAPI and imaged on Axioskop-2 with Axiovision image analysis software. For pupal digestive system staining, 4-day-old pupae were dissected out of their pupal cases and fixed in 1% glutaraldehyde in 1x PBS overnight at 4 °C. The digestive system was dissected in 1x PBS at room temperature, washed 3x10 min in PBS, and stained with 0.2% X-gal in staining buffer for 15 min (*DJ717-gal4*, *DJ626-gal4*) or 1 h (*DJ628-gal4*). Mounting and visualization were performed the same as the adult digestive systems.

### 2.5. 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.

### 2.6. 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 h, the flies were transferred to a fresh vial. After another 24 h, the blue fecal spots on the vial were counted.

### 2.7. Haematoxylin and eosin staining

Flies were decapitated and heads were fixed in 4% paraformaldehyde in 1 × PBS for 3 h at 4 °C. Heads were washed 3 times for 10 min in 1 × PBS and then were incubated in 30% sucrose in 1 × PBS at 4 °C overnight. Heads were mounted in Tissue-Tek OCT Compound (Sakura, Toyko, Japan), snap frozen, and sectioned at 5 µm. Sections were stained with haematoxylin (VWR, West Chester, PA) and eosin (VWR), mounted in Permount (Electron Microscopy Sciences, Hatfield, PA), and imaged on an Axioskop-2 microscope (Zeiss, Thornwood, NY) at 10x with Axiovision image analysis software (Zeiss). Neurodegeneration was scored by the presence of holes in intact brain

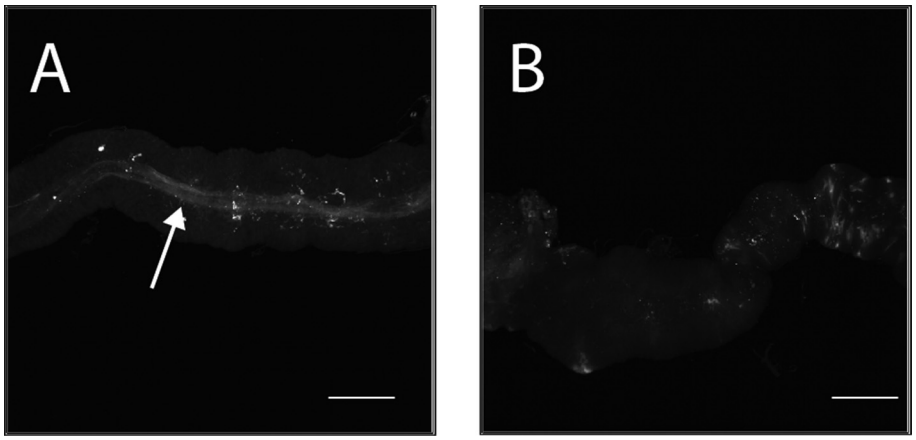


**Fig. 1.** Staining of the PM following calcofluor feeding. Two-day old heterozygous $drd^{lwf}/FM7a$ (A) and homozygous $drd^{lwf}/drd^{lwf}$ (B) females were fed calcofluor to stain the PM (arrow in A). Midguts were then dissected and imaged. Scale bars: 100 μm.

tissue. Multiple sections of a single brain were viewed and the presence or absence of holes was consistent throughout the whole tissue.

### 2.8. Construction of drd-gal4 driver

The gal4 driver line *yw drd-gal4/FM7a* was made through Recombination-Mediated Cassette Exchange of a *T2A-gal4* "Trojan" cassette into the MiMIC insertion $drd^{MI15121}$ (Bellen et al., 2011; Diao et al., 2015; Venken et al., 2011). $y^1$ $w^*$ $Mi\{y^{+mDint2} = MIC\}drd^{MI15121}/FM7h$; $P\{w^{+mC} = loxP(Trojan\text{-}GAL4.1)\}12B$ females were obtained from a stock generated by crossing $y^1$ $w^*$ $Mi\{y^{+mDint2} = MIC\}drd^{MI15121}/FM7h$ x $y^1$ $w^*$; $P\{w^{+mC} = loxP(Trojan\text{-}GAL4.1)\}12B$; $Dr^1/TM3$, $Sb^1$. These females were crossed with $y^1$ $w^{67c23}$; $CyO/+$, $P\{w^{+mC} = Crew\}DH1/+$; $M\{vas\text{-}int.Dm\}ZH\text{-}102D/+$ males that were generated by crossing $y^1$ $w^{67c23}$; $sna^{Sco}/CyO$, $P\{w^{+mC} = Crew\}DH1$ x $y^1$ $M\{3xP3\text{-}RFP.attP\}ZH\text{-}2A$ $w^*$; $M\{vas\text{-}int.Dm\}ZH\text{-}102D$. Individual yellow$^-$ male progeny were crossed with $C(1)DX$, $y^1$ $f^1$ and genotyped to confirm the presence and orientation of the *T2A-gal4* insertion. The PCR primers used for genotyping were ATTTCAATCGGTCGCTGACT and GCTCTCCTCGCTGCTG. One confirmed *yw drd-gal4* chromosome was then balanced over *FM7a*.

### 2.9. Synthesis of the UAS-drd (III) line

The *drd* coding sequence was amplified (KOD Hotstart polymerase, EMD Millipore, Billerica, MA) in a two-step amplification from a previously constructed *UAS-drd* plasmid using the primers AAAAAGCAG GCTGCATGTCGCGTATGTCGCATAT and AGAAAGCTGGGTTCTAATCC GAGTGCGGATGAT and the Gateway attB adapter primers GGGGACA AGTTTGTACAAAAAAGCAGGCT and GGGGACCACTTTGTACAAGAAA GCTGGGT. The resulting PCR product was cloned first into the Gateway donor vector pDONR221 (Thermo Fisher Scientific, Waltham, MA) and then into the expression vector pBID-UASC-G (gift from Dr. Brian McCabe (Addgene plasmid # 35,202)) (J.-W. Wang et al., 2012). The transgene was integrated into the P{CaryP}attP2 site on chromosome 3L (Genetivision Corp., Houston, TX).

### 2.10. Visualization of GFP reporter expression

Cardias were dissected in PBS or Insect Ringers and stained for 30 min in 1 μg/mL DAPI (Biotium, Fremont, CA) in PBS-T. After rinsing, samples were imaged on a Nikon A1 Confocal Microscope (Nikon, Tokyo, Japan) with NIS-Elements AR software (Nikon).

### 2.11. Statistics and data analysis

Data were graphed and analyzed using GraphPad Prism v6 for Windows (GraphPad Software, San Diego, CA, www.graphpad.com). A non-parametric test was used for the defecation data in Fig. 8 because the RNAi dataset was not normally distributed, as determined by a D'Agostino & Pearson omnibus normality test. Survival curves were compared with a Mantel-Cox log-rank test.

## 3. Results

### 3.1. drd mutants lack a peritrophic matrix

We have previously reported defective gut function in *drd* mutants, as indicated by enlarged crop volumes, slow movement of food into the midgut, and decreased defecation (Blumenthal, 2008; Peller et al., 2009). We now find 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 (Fig. S1). A PM was always observed (more than 50 dissections) in adult wild-type flies or females heterozygous for the severe alleles $drd^1$ or $drd^{lwf}$, but we never observed a PM in at least 50 dissections of adult $drd^1$ or $drd^{lwf}$ hemizygous males or homozygous females (aged 2–7 days post-eclosion). In contrast, formation of the larval PM did not appear to be affected by mutation of *drd*, as we observed a PM in 16/16 male third-instar $drd^{lwf}$ mutants by gross dissection. To confirm the absence of the PM in adult *drd* mutants, we imaged dissected midguts of *drd* mutants after feeding with the chitin-binding dye calcofluor. Wildtype midguts showed a bright, clear, structured blue staining down the midgut, indicating the presence of a PM, while *drd* mutant midguts showed only weak, diffuse blue staining (n = 10 total two-day old $drd^{lwf}$ and $drd^1$ heterozygous females and 7 homozygous females, Fig. 1). Because *drd* mutant flies have a defect in the movement of food into their midgut, the absence of calcofluor staining in mutants could have resulted from a failure of the dye to reach the PM. To control for this possibility, dissected midguts from wild-type and *drd* mutant flies were incubated in calcofluor, and again, a clearly stained PM was observed in the wild-type but not the mutant midguts (n = 8 total two-day old $drd^{lwf}$ and $drd^1$ heterozygous females and 17 homozygous females, Fig. S2). Finally, the PM was visualized in semi-thin sections. As shown in Fig. 2A and 2B, the PM is visible in cross-sections of the midgut of $drd^{lwf}$ heterozygous females but not $drd^{lwf}$ homozygotes. In addition, longitudinal sections of the cardia of heterozygotes show the PM associated with the anterior midgut cells and proceeding down the midgut lumen, while in homozygotes the cardia is severely distended and no recognizable PM is present (Fig. 2C and 2D).

### 3.2. drd is expressed in the cardia

Because the cardia is the site of PM synthesis in *Drosophila*, we hypothesized that *drd* is expressed in the cardia. To determine the

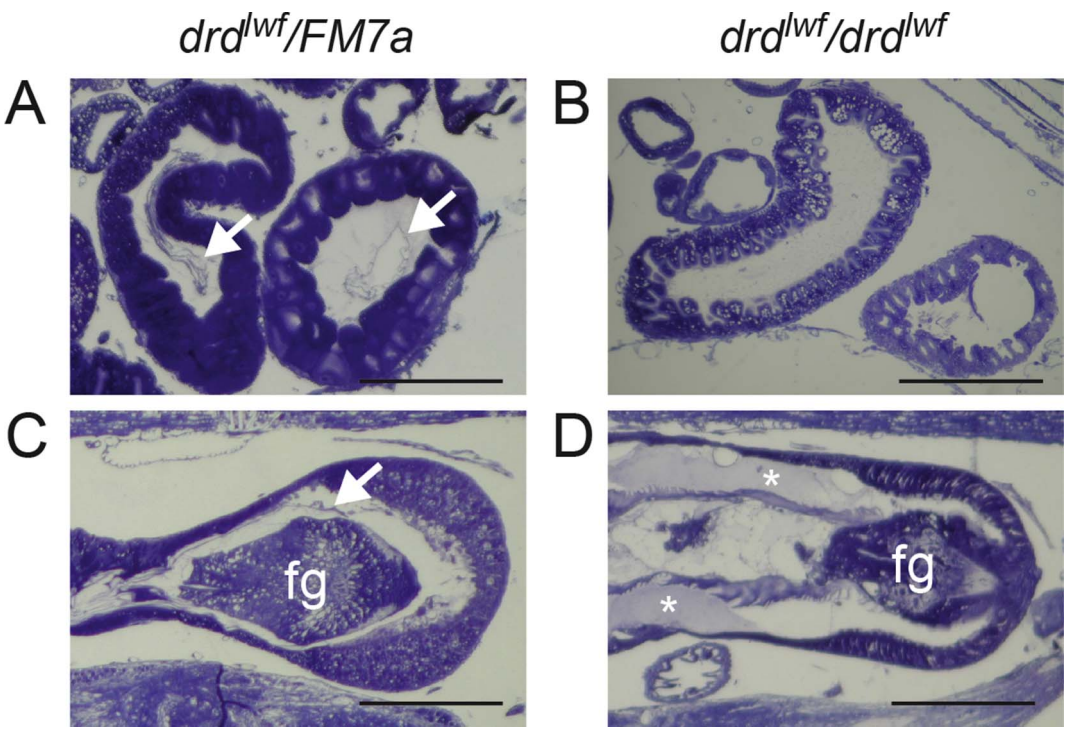


**Fig. 2.** Histology of *drd* mutant midguts. One-day old $drd^{lwf}/FM7a$ (A, C) and $drd^{lwf}/drd^{lwf}$ (B, D) females. Arrows indicate the PM in cross-sections of the midgut (A, B) and longitudinal sections of the cardia (C, D). In C and D, the cardia is oriented with the anterior to the right, and the interior foregut section is marked (fg). Note the diffuse grey material posterior to the cardia in the homozygous mutant (asterisks). Similar results were observed in sections from three heterozygotes and three homozygotes. Scale bars: 100 μm.

endogenous expression pattern of *drd*, we used the gal4-UAS gene expression system. Using recombination-mediated cassette exchange (see methods), we constructed a *drd-gal4* driver in which expression of the yeast gal4 transcriptional activator is controlled by the regulatory elements of the *drd* gene. This gal4 driver can then be used to induce expression of any transgene attached to the UAS DNA sequence (Brand and Perrimon, 1993). Note that as a result of the gal4 insertion into the *drd* gene, the *drd-gal4* driver is itself a *drd* null mutant. However, when this insertion was used to drive *drd* expression from a *UAS-drd* transgene, the resulting flies all had PMs and showed 100% survival for the first 40 days post-eclosion (Fig. 3 and Table 1). This result indicates that the spatiotemporal expression pattern of *drd* defined by the *drd-gal4* transgene is sufficient to rescue both PM formation and adult survival. We then combined this *drd-gal4* driver with a *UAS-GFP* reporter and observed the resulting GFP expression in the recurrent wall of the anterior cardia of both pupal and adult flies (Fig. 4).

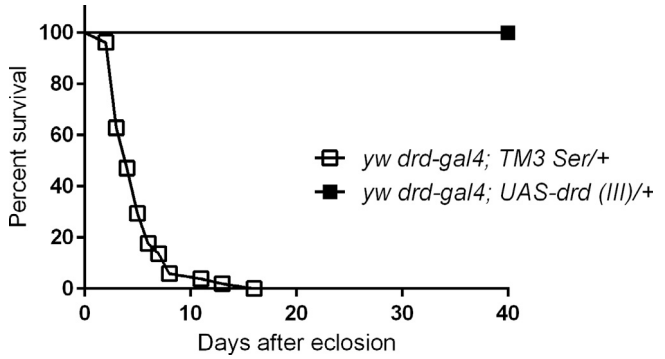


**Fig. 3.** Complete rescue of adult survival with the *drd-gal4* driver. Complete rescue of adult survival in *drd* mutants was observed when expression of the *UAS-drd* transgene was driven by *drd-gal4* (filled squares) compared with mutants lacking *UAS-drd* (open squares). n = 51–55 male flies per genotype. Complete rescue was also observed in females using the *drd-gal4* driver and the second chromosome *UAS-drd* transgene (Fig. S3).

**Table 1**
Presence of the PM following knockdown or rescue of *drd* expression with various gal4 drivers.

| Genotype | PM Present |
|---|---|
| *drd-gal4; UAS-drd (III)/+* | 25/25 |
| *drd-gal4; TM3 Ser/+* | 0/15 |
| $drd^{GD15915}$*/+; DJ626/+* | 2/18 |
| *CyO/+; DJ626/+* | 18/18 |
| $drd^{GD3367}$*/+; DJ626/+* | 1/18 |
| *CyO/+; DJ626/+* | 15/15 |
| $drd^{GD15915}$*/DJ717* | 0/13 |
| *DJ717/CyO* | 13/13 |
| $drd^{GD3367}$*/DJ717* | 2/18 |
| *DJ717/CyO* | 14/14 |
| $drd^{GD15915}$*/+; DJ628/+* | 16/16 |
| *CyO/+; DJ628/+* | 17/17 |
| $drd^{GD3367}$*/+; DJ628/+* | 15/15 |
| *CyO/+; DJ628/+* | 14/14 |
| $drd^{lwf}$*; UAS-drd/+; DJ626/+* | 17/17 |
| $drd^{lwf}$*; CyO/+; DJ626/+* | 0/9 |
| $drd^{lwf}$*; UAS-drd DJ717/DJ717* | 0/13 |
| $drd^{lwf}$*; DJ717/CyO* | 0/13 |

Each pair of genotypes represents either knockdown or rescue flies (top line) and sibling controls (bottom line). The first and last two pairs are rescue experiments with the *drd-gal4*, *DJ626*, and *DJ717* drivers, respectively, while the second through seventh pairs are knockdown experiments with the *DJ626*, *DJ717*, and *DJ628* drivers and two different *drd* RNAi transgenes. Presence or absence of the PM was assessed by gross dissection in two-day old male flies. Blank rows indicate sibling controls. The totals for each phenotype include progeny from at least two independent crosses.

### 3.3. *drd* expression in the cardia is necessary for peritrophic matrix formation and normal defecation rate

To determine the relationship between *drd* expression in the cardia

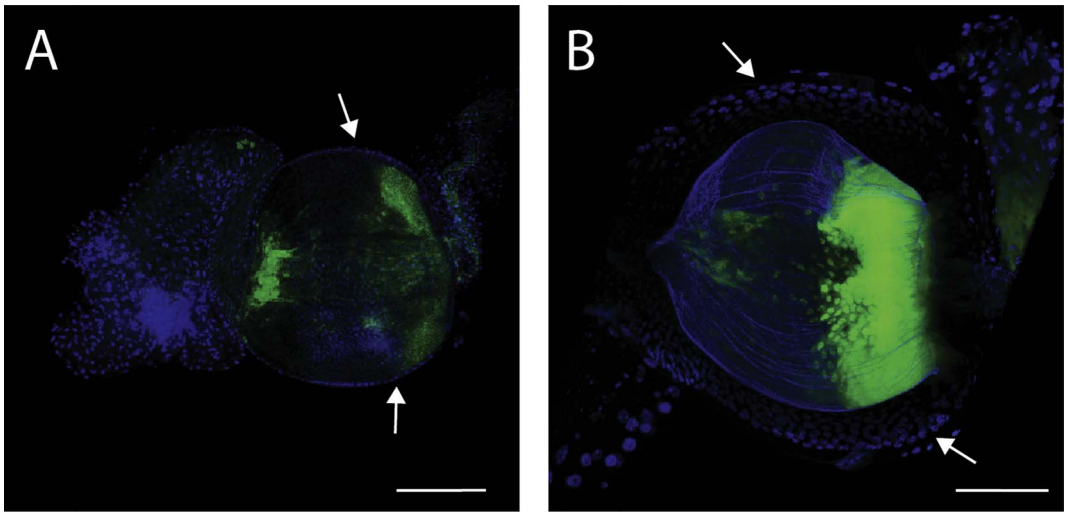


**Fig. 4.** Expression of *drd* in the cardia. Images are shown of a day four pupa (A) and a two-day old adult (B). Both tissues were from *yw drd-gal4/w; UAS-GFP.nls/+* females in which the expression of a nuclear GFP reporter was driven by *drd-gal4*, and nuclei were counterstained with DAPI. Images are oriented with the anterior end of the cardia to the right. Arrows indicate the nuclei of the outer, midgut layer of the cardia. Scale bars: 100 μm (A), 50 μm (B).

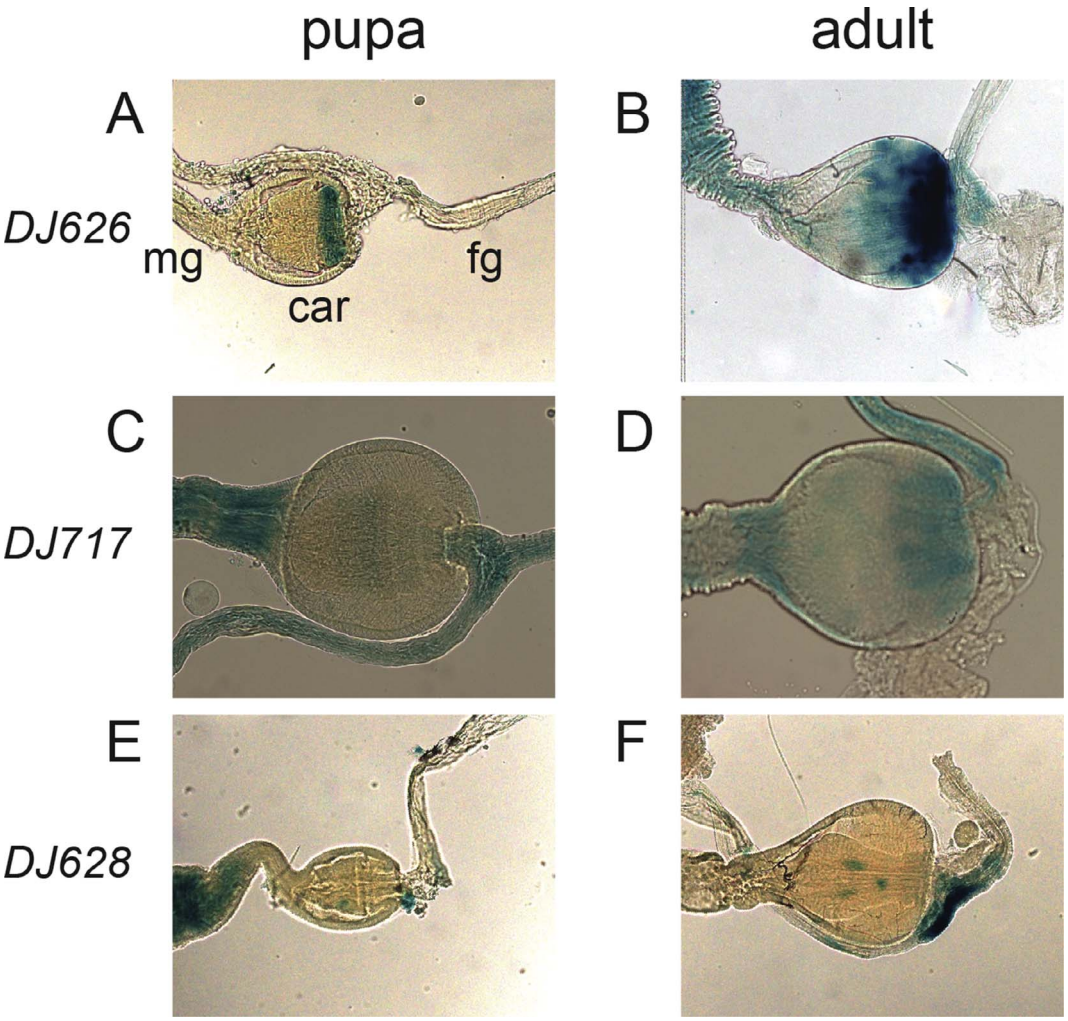


**Fig. 5.** Expression patterns of gal4 drivers in the cardia. Expression is shown of the *DJ626* (A, B), *DJ717* (C, D) and *DJ628* (E, F) enhancer traps in the cardia of day 4 pupae (A, C, E) and two-day old adults (B, D, F). Expression was observed by driving a *UAS-LacZ* reporter transgene followed by X-gal staining. All images are oriented with the anterior end of the cardia to the right. fg: foregut, car: cardia, mg: midgut.

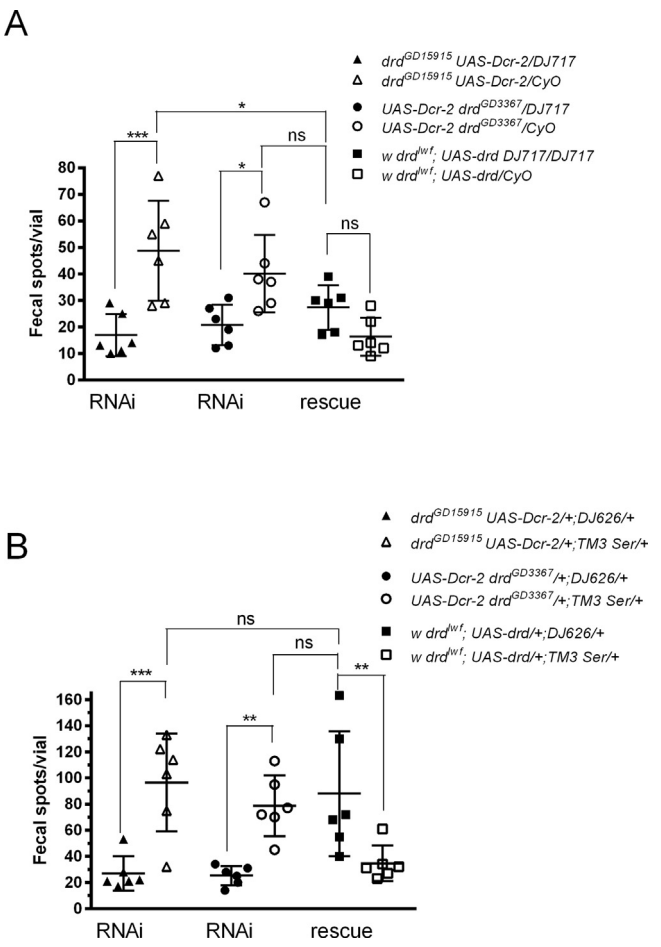


**Fig. 6.** Effect on defecation of knocking down or rescuing *drd* expression with the *DJ717* and *DJ626* drivers. While RNAi with either *DJ717* (A) or *DJ626* (B) (filled triangles and circles) reduced defecation compared to controls lacking the gal4 driver (open triangles and circles), only *DJ626* was able to drive significant rescue of defecation on a *drd* mutant background (filled squares) compared to controls lacking the gal4 driver (open squares) (1-way ANOVA with Bonferroni's multiple comparison test). n = 6 vials per genotype, error bars represent SD.

and the gut-related phenotypes of *drd* mutant flies, we examined the effects of manipulating *drd* expression with three gal4 drivers with differing levels of expression in the cardia: *DJ626*, *DJ717*, and *DJ628* (Seroude, 2002). To visualize their expression patterns, each gal4 driver was used to drive expression of a *UAS-LacZ* reporter, and *LacZ* expression was visualized by X-gal staining. The *DJ626* driver showed robust expression in the anterior cardia of both late pupae and young adults, the *DJ717* driver showed mild expression in the cardia only in the adult, and the *DJ628* driver was not expressed in the cardia (Fig. 5). Importantly, both *DJ626* and *DJ717* are also expressed in the respiratory tracheae ((Sansone and Blumenthal, 2013) and data not shown).

Each of the three drivers was then used to knock down *drd* expression on an otherwise wild-type background. We have previously reported that driving *drd* RNAi with *DJ717* results in early adult lethality and neurodegeneration, similar to the phenotypes that are observed when RNAi is driven by the tracheal-specific driver *btl-gal4* (Sansone and Blumenthal, 2013). Further examination of the *DJ717* RNAi flies indicated that they also lacked a PM and had reduced defecation rates (Table 1 and Fig. 6A). Identical phenotypes were observed when *drd* RNAi was induced by two different transgenes, $drd^{GD3367}$ and $drd^{GD15915}$. Driving *drd* RNAi with *DJ626* gave the same results as with *DJ717*: early lethality (Fig. 7A), reduced defecation (Fig. 6B), loss of the PM (Table 1), and neurodegeneration (holes were observed in 13/18 *drd* RNAi vs 0/11 control brains, Fig. S4). In contrast, *drd* RNAi driven by *DJ628* had no effect on adult survival and did not eliminate the PM (Fig. 7B and Table 1).

When the *DJ717* and *DJ626* drivers were used, in combination with *UAS-drd*, to rescue *drd* expression in otherwise *drd* mutant flies, we observed a difference between the two drivers. Both drivers rescued neurodegeneration (holes were observed in 0/10 20-day old *DJ717* rescue and 0/7 20-day old *DJ626* rescue brains, Fig. S4), and both only partially rescued adult survival, although *DJ626* had a stronger effect (Fig. 7C and D). The partial rescue of adult survival did not appear to be due to the level of expression of the gal4 drivers, as rescue was not enhanced by the addition of a second copy of the driver or UAS transgene (Fig. 7C and D). Only *DJ626* rescued defecation rate and the presence of the PM; flies in which *drd* expression was rescued by *DJ717* lacked a PM and had a defecation rate no different from mutant controls (Fig. 6 and Table 1). Thus, the ability of these two drivers to rescue the gut-related phenotypes of *drd* mutants is correlated to the level of their expression in the cardia.

### *3.4. Continuous drd expression in the adult is necessary for PM formation but the PM is not necessary for adult survival or defecation rate*

The results described above show a correlation among the phenotypes of reduced defecation, lack of an adult PM, and survival beyond approximately 30 days post-eclosion. A causal relationship among the phenotypes would make sense if the absence of a PM impeded food movement through the gut or resulted in starvation by some other mechanism. However, we have previously reported that *drd* expression during metamorphosis, but not in adults, is necessary and sufficient for adult survival (Sansone and Blumenthal, 2012). Because the PM is synthesized continuously during adulthood but presumably has no function in the non-feeding pupae, this result suggests either that *drd* expression in the adult is not required for PM synthesis or that the PM is not required for adult survival. To distinguish between these possibilities, we repeated three previous experiments on life stage-specific knockdown and rescue of *drd* expression and assayed for the presence of the PM and, when appropriate, for defecation rate. In experiment one, Adult Knockdown, *drd* expression was knocked down globally via heat shock-induced RNAi beginning on the day of eclosion, a treatment that has no effect on adult lifespan (Sansone and Blumenthal, 2012). After seven days of heat shock, flies in which *drd* was knocked down with the $drd^{GD3367}$ transgene lacked a PM but showed normal defecation rates compared to sibling controls (Table 2 and Fig. 8). When RNAi was induced with $drd^{GD15915}$, most flies still had a PM after 7 days, but lost the PM with longer heat-shock (Tables 2 and S1); accordingly, only $drd^{GD3367}$ was used in the subsequent experiment. In experiment two, Pupal Knockdown, flies were heat-shocked to knock down *drd* only during metamorphosis, which results in early adult lethality. Three days after eclosion, these short-lived flies did have a PM (Table 2). Finally, in experiment three, Pupal Rescue, *drd* was rescued by heat shock on a *drd* mutant background only during metamorphosis. Seven days after eclosion, these adult flies lacked a PM (Table 2) but, as previously reported, showed rescue of the adult lethal phenotype. For the Pupal Knockdown and Pupal Rescue experiments, we did not measure defecation rate because the experimental flies in the former experiment and the control flies in the latter experiment did not survive long enough to allow comparisons between sibling genotypes. Taken together, these results indicate that *drd* expression is only necessary during adulthood for adult PM formation and surprisingly, that the presence of a PM is not necessary either for adult survival or for normal rates of defecation.

## 4. Discussion

Here, we report a novel and unique gut phenotype of *drd* mutants: the absence of a PM. To be more precise, we have shown that adult *drd*

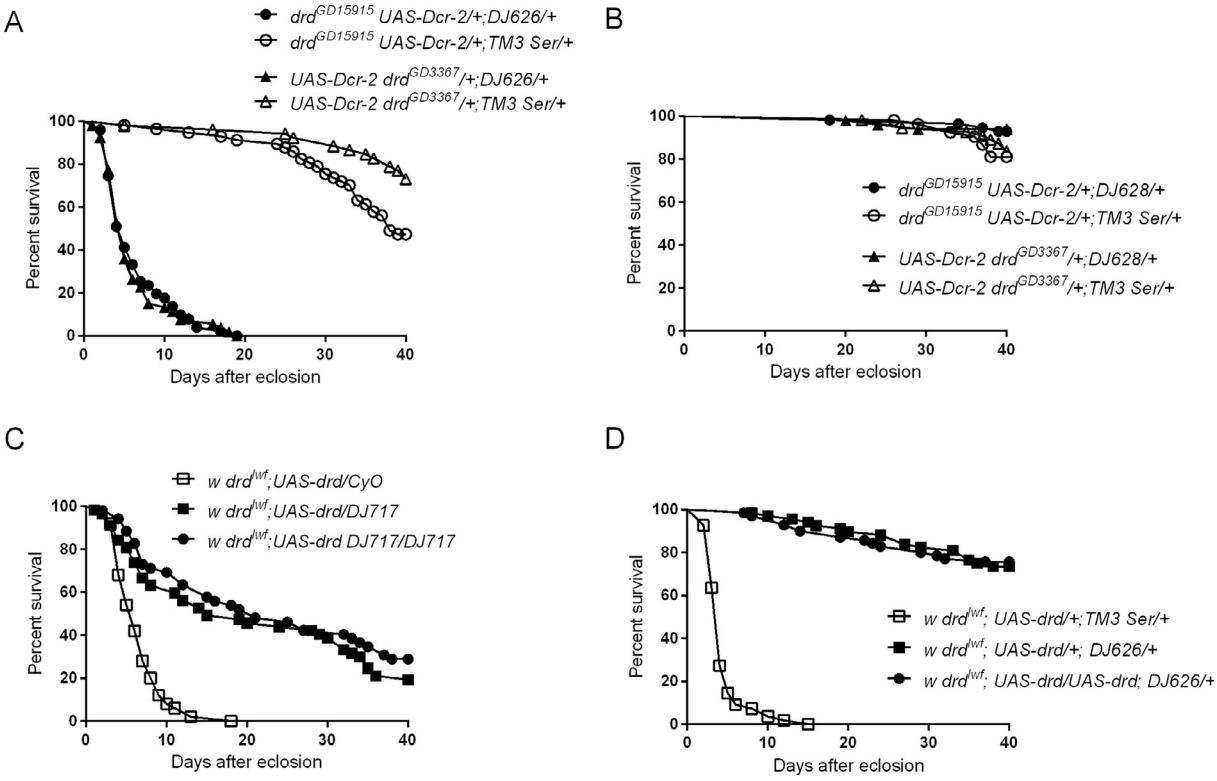


**Fig. 7.** Effect on adult survival of knocking down and rescuing *drd* expression with gal4 drivers. Survival curves are shown following knockdown of *drd* with the *DJ626* (A) and *DJ628* (B) drivers (filled symbols are knockdown flies and open symbols are controls lacking the gal4 driver) and rescue of *drd* expression on a *drd* mutant background with the *DJ717* (C) and *DJ626* (D) drivers (open squares are *drd* mutants lacking the gal4 driver, filled squares are rescue flies, and filled circles are rescue flies with two copies of the gal4 driver (C) or two copies of the *UAS-drd* transgene (D)). n = 50–68 male flies per genotype.

**Table 2**
Adult expression of *drd* is required for PM formation.

| Experiment | Genotype | PM Present |
|---|---|---|
| Adult Knockdown | *UAS-Dcr-2 drd$^{GD3367}$/Hsp70-gal4* | 0/27 |
| | *UAS-Dcr-2 drd$^{GD3367}$/CyO or Hsp70-gal4/CyO* | 19/19 |
| | *UAS-Dcr-2 drd$^{GD15915}$/Hsp70-gal4* | 20/25 |
| | *UAS-Dcr-2 drd$^{GD15915}$/CyO or Hsp70-gal4/CyO* | 17/17 |
| Pupal Knockdown | *UAS-Dcr-2 drd$^{GD3367}$/Hsp70-gal4* | 13/13 |
| | *UAS-Dcr-2 drd$^{GD3367}$/CyO* | 15/15 |
| Pupal Rescue | *w drd$^{lwf}$; UAS drd/Hsp70-gal4* | 1/16 |
| | *w drd$^{lwf}$; UAS drd/CyO* | 0/1 |

The presence of the PM was scored in three experiments designed to knock down or rescue *drd* expression during either metamorphosis or adulthood (see text for details). In the Adult Knockdown experiment, the experimental (knockdown) flies are *UAS-Dcr-2 drd$^{GD3367}$/Hsp70-gal4* and *UAS-Dcr-2 drd$^{GD15915}$/Hsp70-gal4,* while the sibling controls lacking either the RNAi transgene or the gal4 driver are *UAS-Dcr-2 drd$^{GD3367}$/CyO* or *Hsp70-gal4/CyO* and *UAS-Dcr-2 drd$^{GD15915}$/CyO* or *Hsp70-gal4/CyO*. In the Pupal Knockdown experiment, the experimental (knockdown) flies are *UAS-Dcr-2 drd$^{GD3367}$/Hsp70-gal4,* and the sibling controls lacking the driver are *UAS-Dcr-2 drd$^{GD3367}$/CyO*. In the Pupal Rescue experiment, the experimental (rescue) flies are *w drd$^{lwf}$; UAS drd/Hsp70-gal4,* and the mutant sibling controls lacking the driver are *w drd$^{lwf}$; UAS drd/CyO*. The two knockdown experiments included both males and females, while the Pupal Rescue experiment only utilized males. The totals for each phenotype except the last include progeny from at least two independent crosses. Only a single fly was tested for the final phenotype because all other controls died in the first seven days post eclosion.

mutants lack a detectable PM by gross dissection, chitin staining, and histology, while the PM is still present in larval *drd* mutants. However, 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). It is possible that *drd* mutants lack only the first layer, and that the material that would normally make up the remaining layers either fails to condense into a discrete structure (possibly consistent with the

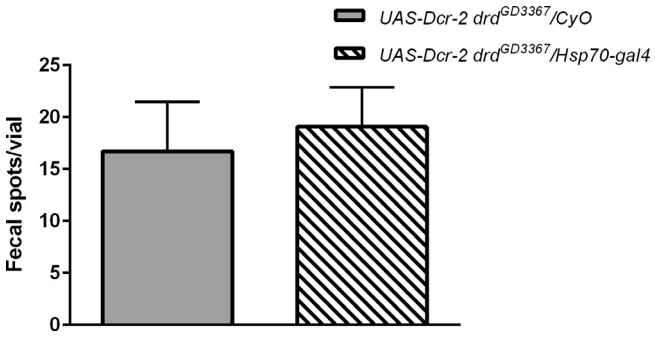


**Fig. 8.** Knockdown of *drd* expression in the adult does not affect defecation rate. Defecation rate was measured in flies following heat-shock for the first seven days post eclosion (see text for details). No difference was seen between knockdowns (striped bar, n = 32 vials) and sibling controls (solid bar, n = 14 vials), p = .14, Mann-Whitney test. Error bars represent SD.

lightly stained material filling the cardia in Fig. 2D) or forms a structure that is not detectable by either histology or gross dissection. Consistent with a specific effect on a single chitinous layer of the PM, *drd* expression in the cardia appears to be localized to a region just anterior to the foregut/midgut transition, termed zones 2 and 3, which is where the first layer of the PM is synthesized (King, 1988).

The DRD protein is proposed to function as an acyltransferase, based on sequence homology to biochemically characterized prokaryotic proteins, but no biochemical function for DRD or any related eukaryotic protein has been reported (Blumenthal, 2008). The protein contains multiple putative membrane-spanning domains and has been localized to an unidentified intracellular compartment (Kim et al., 2012). Based on its location and structure, it is possible that DRD is required in the secretory pathway of cardia epithelial cells for the posttranslational modification of a PM structural protein or synthetic enzyme.

In our previous study, we used tracheal-specific knockdown and rescue of *drd* expression to identify the suite of phenotypes associated with a lack of *drd* expression in the tracheae, namely early adult lethality and neurodegeneration but not gut dysfunction (Sansone and Blumenthal, 2013). In the current work, we show that expression of *drd* in the pattern defined by the *DJ626* driver, which is expressed in both the tracheae and the anterior cardia, was both necessary and sufficient for both the "tracheal" phenotypes of early lethality and neurodegeneration and the "gut" phenotypes of reduced defecation and the absence of a PM. The pattern defined by the *DJ717* driver, which is expressed at lower levels in the cardia than *DJ626* and is also expressed in the tracheae, was both necessary and sufficient for the tracheal phenotypes and was effective in creating the gut phenotypes in knockdown experiments but not in rescuing them. These data suggest that the gut phenotypes are related to *drd* expression in the cardia. However, both *DJ626* and *DJ717* are expressed in other tissues and other parts of the gut, and the absence of a driver specific to the anterior cardia precludes a direct test of this hypothesis.

Using temporal control of *drd* expression, we can separate the two gut phenotypes, with the presence of a PM associated with *drd* expression in the adult and normal defecation associated with *drd* expression during pre-adult development. This result was surprising in two respects. First, it shows that the presence of the PM is not required for movement of ingested food through the fly's digestive tract. Second, it demonstrates that *drd* plays at least two different roles in the development and maintenance of the *Drosophila* gut and that the roles occur during different stages of the organism's life cycle. As further evidence for the pleiotropic nature of *drd* function, we find that rescuing *drd* expression with the *DJ626* driver did not fully rescue adult lethality, while rescuing expression with the *drd-gal4* driver did achieve a complete rescue in survival. Thus, it appears that besides neurodegeneration and reduced food movement through the gut, *drd* mutants have at least one more physiological defect that eventually leads to adult lethality. The *drd-gal4* driver will be an important tool in identifying further *drd* phenotypes by revealing the full expression pattern of *drd*.

By knocking down *drd* expression only during adulthood, we can create flies that lack a PM, and we have previously reported that this treatment has no effect on adult survival (Sansone and Blumenthal, 2012). However, in order to sustain *drd* knockdown in the adult, we must maintain flies at 30 °C. Under these conditions, both knockdown and control flies began dying around 15 days post eclosion and exhibited 100% mortality by day 40. Kuraishi et al. have reported that flies mutant for the PM protein Crystallin (DCY), which have a leaky PM, begin dying around day 20 after eclosion and show approximately 50% mortality by day 40 (Kuraishi et al., 2011). Due to the temperature-induced lethality observed in our adult knockdown flies, we cannot exclude the possibility that the PM is required for adult survival beyond three weeks, consistent with the results of Kuraishi et al.

It has previously been reported that a "PM-less" phenotype can be created in the larvae of several insect species through feeding with either Calcofluor or chitinase (Bolognesi et al., 2001; Rao et al., 2004; P. Wang and Granados, 2000). However, the *drd* mutant flies are the first reported *Drosophila* strain with a genetically ablated PM. Interest in the adult insect PM has increased recently due to its theorized role in immunity, microbiome maintenance, and virulence propagation and its identity as a potential target for pesticides (Abraham et al., 2017; El-Bassiony et al., 2016; Shi et al., 2016). Studying gut function in *drd* mutants and knockdowns is complicated by the expression and function of *drd* in multiple tissues and the many severe phenotypes that are independent of the PM. However, this mutation causes a more complete phenotype than "leaky" PM models (Kenmoku et al., 2016; Kuraishi et al., 2011), and we anticipate that the *drd* mutant fly will be an invaluable tool to better study the physiological roles and developmental pathways leading to the synthesis of the PM.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jinsphys.2018.01.007.

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