Transcriptome Analysis of Drosophila melanogaster Third Instar Larval Ring Glands Points to Novel Functions and Uncovers a Cytochrome p450 Required for Development

Danielle Christesen, Ying Ting Yang, Jason Somers,1 Charles Robin, Tamar Sztal,2 Philip Batterham, and Trent Perry3
School of Biosciences, Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, Parkville, Victoria 3010, Australia

ABSTRACT In Drosophila melanogaster larvae, the ring gland (RG) is a control center that orchestrates major developmental transitions. It is a composite organ, consisting of the prothoracic gland, the corpus allatum, and the corpora cardiaca, each of which synthesizes and secretes a different hormone. Until now, the RG’s broader developmental roles beyond endocrine secretion have not been explored. RNA sequencing and analysis of a new transcriptome resource from D. melanogaster wandering third instar larval RGs has provided a fascinating insight into the diversity of developmental signaling in this organ. We have found strong enrichment of expression of two gene pathways not previously associated with the RG: immune response and fatty acid metabolism. We have also uncovered strong expression for many uncharacterized genes. Additionally, RNA interference against RG-enriched cytochrome p450s Cyp6u1 and Cyp6g2 produced a lethal ecdysone deficiency and a juvenile hormone deficiency, respectively, flagging a critical role for these genes in hormone synthesis. This transcriptome provides a valuable new resource for investigation of roles played by the RG in governing insect development.

KEYWORDS ecdysteroidogenesis immune response cytochrome p450 Halloween genes molting

Endocrine control of insect development is a complex symphony, with hormones produced in overlapping waves that determine the timing and nature of each developmental transition. In Drosophila melanogaster larvae, an endocrine organ, the ring gland (RG), is the control center that produces many of these hormones to orchestrate larval molts and the larval-pupal transition.

Located anterior to the larval central nervous system (CNS), the RG is a composite organ consisting of three different subunits (King et al. 1966) (see Figure 1), each of which synthesizes and secretes a different hormone. The prothoracic gland (PG) is the major subunit of the RG, both by size and cell number (King et al. 1966). The PG synthesizes the insect molting hormone ecdysone (Vogt 1943; Wigglesworth 1954), which is released into the hemolymph for conversion to its active form 20-hydroxyecdysone (20E) at peripheral target tissues (Petryk et al. 2003). 20E directly triggers major developmental events in the larva, and its precursor ecdysone is secreted by the PG cells in clearly defined pulses to provide temporal control of these events; there is a single pulse prior to each larval molt, prior to pupariation, and at the commencement of metamorphosis (Riddiford 1993; reviewed in Baehrecke 1996; Thummel 2002; Ou and King-Jones 2013).

The second-largest RG subunit is the corpus allatum (CA) (King et al. 1966). Throughout the first and second larval instars, the CA cells synthesize and secrete juvenile hormone (JH), which determines the nature of all 20E-induced transitions (Williams 1961; Bownes and Rembold 1987; Sliter et al. 1987). In the presence of JH, 20E will always trigger a larval-larval molt (Riddiford 1970). Upon attainment of critical weight early in the third larval instar, JH production at the CA ceases, allowing...
20E to initiate the changes in gene expression required for metamorphosis (reviewed in Berger and Dubrovsky 2005; Rewitz et al. 2013).

The third and smallest RG subregions are the corpora cardiaca (CC), found paired at the base of each SG lobe (King et al. 1966). The CC cells are heavily involved in glucose regulation, being the primary site of adipokinetic hormone (Akh) production in the larva (Kim and Rulifson 2004). Akh is a peptide hormone that is functionally equivalent to mammalian glucagon; it is active in the larval fat body, where it triggers mobilization of lipids and carbohydrates into the hemolymph (Bharucha et al. 2008).

This transcriptome analysis of wandering third instar larvae encompasses all three RG subregions. There are a number of questions surrounding the role of the RG subregions that are addressed. First, there are a number of genes in the ecdysteroidogenesis pathway that are yet to be identified [known as the "Black Box" genes (reviewed in Grieneisen 1994; Rewitz et al. 2006; Niwa and Niwa 2014)]. Many of the known ecdysteroidogenesis reactions are catalyzed by cytochrome P450s (CYPs) (Chavez et al. 2000; Warren et al. 2002, 2004; Petryk et al. 2003; Niwa et al. 2004; Ono et al. 2006) so CYPs expressed in the RG would be candidate Black Box genes. Second, this transcriptomic analysis provides the opportunity to clarify ecdysteroidogenesis regulatory pathways of D. melanogaster. A multitude of tropic and static factors bind in the PG cells to provide tight temporal control of ecdysteroidogenesis (see Figure 2) (reviewed in Huang et al. 2016; Marchal et al. 2010; and Yamanaka et al. 2013); however, some components within these pathways have been investigated only in lepidoptera. Third, while ecdysteroidogenesis is recognized as the primary function of the PG, there is ultrastructural evidence from D. melanogaster that suggests the PG cells may be performing other roles, particularly in late larval development before the PG cells regress (Dai and Gilbert 1991).

Using RNA-seq, we have gained a fresh insight into the range of genes expressed in the D. melanogaster wandering third instar RG. We identified 2462 genes significantly enriched in the RG relative to the CNS. As RG-enriched genes include those involved in hormone synthesis, but there were also genes involved in the immune response, and many (1310) uncharacterized genes. One of the RG-enriched CYP genes, Cyp6u1, was knocked down in the PG using RNA interference (RNAi). This produced a lethal low ecdysone phenotype, flagging a critical role for this gene in development. We also provide a comparison between our data and a recently published RG resource obtained by microarray (Ou et al. 2016). As the first complete RG transcriptome, examination of the many highly enriched genes identified in this study may ultimately reveal entirely novel function(s) of the RG subregions.

**Figure 1** Position and substructure of the D. melanogaster third instar larval RG. (A) GFP expression driven by 5′ phm-GAL4 indicates the position of the RG in the whole larva. It is located dorso-anterior to the larval central nervous system. (B) The RG is a composite endocrine organ consisting of three distinct subregions: the prothoracic gland, the corpus allatum, and the paired corpora cardiaca. Each subregion synthesizes a different hormone.

**Figure 2** Regulation of ecdysteroidogenesis. A huge range of factors influence the ecdysteroidogenic output of the PG cells. PTTH is the major tropic regulator. When PTTH binds its receptor Torso, this activates a Ras-Raf-ERK pathway and a Ca2+-dependent pathway. Other tropic pathways include ILP signaling, TOR signaling, serotonin signaling, and NO signaling, plus activin upregulates INR and Torso. JH and 20E can both downregulate ecdysteroidogenesis. Our knowledge of these regulatory signaling pathways comes from studies in lepidoptera only (italic text), or from studies in both lepidoptera and diptera (bold text). PTTH, prothoracicotropic hormone; Cam-AdCyc, calmodulin-adenylase cyclase; NO, nitric oxide; JH, juvenile hormone; 20E, 20-hydroxyecdysone; ILP, insulin-like peptide. (Adapted from Marchal et al. 2010; Yamanaka et al. 2013).

**MATERIALS AND METHODS**

**Dissection, RNA isolation and sequencing**

RGs were dissected from wandering third instar larvae for two wild-type strains: the reference genome strain y1; cn1 bw1 sp1 (Cel) and Armenia14 (A14) (Perry et al. 2012) (all fly stocks listed in Supplemental Material, Table S1). Dissections were performed in 100% PBS in batches of 10–40 at a time, then pooled into three biological replicates for both Cel and A14; ~80 RGs were pooled to provide the ~1 μg of RNA required for sequencing. Total RNA was extracted using the Reliaprep RNA Cell Miniprep System (Promega), then stored at −80 °C. Total RNA was quality assessed using the 2100 Bioanalyzer (Agilent Technologies), polyA enriched, cDNA libraries prepared, and 100 bp paired-end RNA-seq performed on the Illumina HiSeq2000 system (Australian Genome Research Facility, AGRF). In addition to the reads obtained from the six RG samples, duplicate RNA-seq reads for the Oregon-R wandering third instar CNS were downloaded from the modMINE database (accession: SRX029398) (Contrino et al. 2012). These reads were downloaded in SRA (short read archive) format, and converted to paired end fastq format using the fastq-dump utility included in the NCBI SRA toolkit.
Transcriptome construction and analysis

Paired fastq sequencing reads were aligned to the annotated D. melanogaster reference genome (BDGP release 5) using TopHat 2.0.13 (Trapnell et al. 2012). Expression levels were quantified as FPKM (fragments per kilobase of transcript per million fragments mapped), and differential expression was calculated using Cufflinks 2.2.1 (Trapnell et al. 2012), with options to enable reference annotation based transcript assembly (−g), fragment bias correction (−b), multi-read correction (−u), and increased maximum fragment alignment (−max-bundle-frac). Quality of the samples was confirmed by examining the dataset for expression of transcripts that would indicate contamination (see Figure S1). Gene ontology enrichment analysis was carried out using the Functional Annotation Clustering tool from the Database for Annotation, Visualization and Integrated Discovery (DAVID 6.7) (Huang et al. 2009). Clusters with enrichment scores of at least 1.3 (equivalent to nonlog P < 0.05) were further investigated. Secretome analysis was carried out using Signal P 4.1 (Petersen et al. 2011). A D-score of ≥0.45 was used as the cutoff value to discriminate signal peptides from nonsignal peptides. Flybase (St Pierre et al. 2014) was used to investigate gene function.

RNAi gene knockdown

Using available UAS-dsRNA lines and DNA constructs from the Vienna Drosophila RNAi Center (VDRC) (Dietzl et al. 2007), select RG-enriched CYPs (Cyp4g1, Cyp4d2, Cyp6g2, Cyp6u1, and Cyp6v1) were knocked down. Five UAS-RNAi males were crossed to five virgin GAL4 females to achieve ubiquitous knockdown (tubulin-GAL4) and RG-specific knockdowns (5’ phm-GAL4, PG, 5’ phg-GAL4, CA; Akh-GAL4, CC). All crosses were conducted at 22°C with four replicates. Significance was calculated using a Student’s t-test. Where lethality was observed, crosses were also conducted in cages and 50 first instar larvae were picked into vials (n = 250). To monitor developmental timing, 10 first instar larvae were picked onto grape juice plates (n > 40) and developmental stages scored daily. All fly stocks used are listed in Table S1.

qPCR was used to validate RNAi knockdown of RG-enriched CYPs (Cyp4g1, Cyp4d2, Cyp6g2, Cyp6u1, and Cyp6v1), and to measure expression of the JH-regulated gene Kruppel homolog 1 (Kr-h1) in Cyp6g2 RNAi flies. Virgin tubulin-GAL4 females were crossed to males carrying each UAS-dsRNA construct and males from each of the control lines w1118 and 60100. For each of three biological samples, 10 whole second instar larvae were collected, and RNA was isolated using either the Reliaprep RNA Cell Miniprep System (Promega) (Cyp4g1, Cyp4d2, Cyp6u1, and Cyp6v1) or using TRIzol Reagent (Thermo Fisher Scientific) (Cyp6g2). RNA concentration was measured using the Qubit Fluorometer. cDNA was synthesized from 440 ng RNA using the SuperScriptIII Reverse Transcriptase kit (Invitrogen). qPCR reactions for each biological sample were carried out in triplicate using a Quantifast SYBR Green PCR kit (Qiagen) on the CFX384 Touch Real-Time PCR Detection System (Bio-Rad). The amount of target RNA was normalized to the endogenous controls Rpl32 and CG13220 (Van Hiel et al. 2009) (Cyp4g1, Cyp4d2, Cyp6u1, and Cyp6v1) or Rpl11 and Rpl24 (Cyp6g2 and Kr-h1). mRNA levels were compared between samples using the ΔΔCt method (Bustin et al. 2009) using qbase+ software (Biogazelle). All primer sets used are provided in Table S2, the MIQE checklist is provided in Table S3, and qPCR results are in Figure S2.

Ecdysteroid extraction and ELISA

Ecdysteroids were extracted and quantified following a procedure adapted from Yamanaka et al. (2015). Ten RG–CNS complexes were dissected and rinsed in PBS, then pooled in 300 μl of methanol on ice. The tissue was homogenized by passing through a 23-gauge needle and centrifuged at 4°C for 5 min. Supernatants were collected, and the pellet re-extracted with 300 μl methanol. For hemolymph samples, 4 μl hemolymph was collected from 10 larvae, and mixed with 100 μl of methanol on ice. Samples were vortexed and centrifuged at 4°C for 5 min. All supernatants were stored at −20°C prior to use. Immediately prior to the ELISA, all sample solutions were dried with a SpeedVac concentrator, and dissolved in EIA buffer from the 20-hydroxyecdysone EIA kit (Cayman Chemical). The ELISA assay was performed according to the manufacturer’s instructions. Ecdysteroid levels were normalized to the amount of protein in each sample. Protein levels were measured with a Bradford Protein Assay (Bio-Rad) according to the manufacturer’s instructions.

Data availability

Strains are available upon request. Raw sequence reads and processed data files, including the table of FPKM estimates output by Cuffdiff, are available from the National Center for Biotechnology Gene Expression Omnibus under the accession number GSE76304.

RESULTS AND DISCUSSION

Gene expression in the third instar larval ring gland

Total RNA was extracted from the RGs of Cel and A14 wandering third instar larvae then submitted for RNA-seq (summarized in Table S4). A total of 188,742,322 reads was generated by 100 bp paired-end sequencing using an Illumina HiSeq2000 at the AGRF. These reads were evenly distributed among the six samples, with the sequencing depth ranging from 28,578,817 to 33,621,275 reads. In addition to the reads obtained from the six RG samples, two replicates of RNA-seq reads for the wandering third instar CNS were downloaded from modMINE (Contrino et al. 2012). Given the proximity to the RG, the CNS reads were used to check for any contamination, and for differential gene expression analysis. For all eight samples, overall read alignment rates were very high, ranging from 87.1 to 91.4%. Concordant pair alignment rates were slightly lower, but still well within acceptable limits, ranging from 78.6 to 86.7%.
Cufflinks (Trapnell et al. 2012) was used to calculate the FPKM values. As can be seen in Figure 3, a similar FPKM distribution pattern was found in both RG samples and in the CNS. A large number of genes were very lowly expressed (FPKM < 1), the majority of genes were lowly (1 < FPKM < 10) to moderately expressed (10 < FPKM < 50), and there were fewer genes highly (50 < FPKM < 1000) to extremely highly (FPKM > 1000) expressed (Gelbert and Emmert 2013). Many of the genes in the latter category were ribosomal proteins (see Table S5).

To determine the total number of genes expressed in the RG, a FPKM threshold of one was applied to the dataset (Adrian and Comeron 2013; et al. 2014). Of the 8055 RG genes, differential expression analysis revealed that 2462 of these gene transcripts were significantly enriched in both RG samples relative to the CNS. The degree of enrichment exceeded 100-fold for 40 of these RG-enriched gene transcripts (see Table 1 and Table S7). Among these, 20 were genes of unknown function. The values used to calculate differential expression (CNS vs. RG) were from the Cel samples. Differential expression analysis using the A14 data provided similar results (see Tables S5–10, S12–13). A notable caveat of using the CNS for differential expression analysis is that transcripts may be reported as RG-enriched when in fact they are CNS-depleted relative to other tissues. This must be considered when interpreting our results.

### Ring gland expression of ecdysteroidogenesis genes

Much of what is known about ecdysteroidogenesis comes from a combination of lepidopteran and dipteran studies. This RNA-seq data provides a more complete picture of pathways not fully investigated in *D. melanogaster*. We explored the expression levels of key genes that are either involved in the regulation of ecdysteroidogenesis, or are members of the ecdysteroidogenic pathway (see Table 2 and Table S8). All genes in the central ecdysteroidogenesis pathway were highly expressed, with the exception of *spook* and *shade*. Low expression of

---

**Table 1** Most highly enriched genes in the RG, sorted by FPKM value

| Flybase Symbol | Gene Name                     | FPKM*  | Fold Enrichment* | GO Term | Biological Process                      | Molecular Function                  |
|----------------|-------------------------------|--------|------------------|---------|----------------------------------------|-------------------------------------|
| phm            | Phantom                       | 13,305 | +113.35          | Ec dysone biosynthetic process | Ecdysteroid 25-hydroxylase activity |
| sad            | Shadow                        | 12,617 | +161.98          | Ec dysone biosynthetic process | Ecdysteroid 2-hydroxylase activity  |
| Npc1a          | Niemann-Pick C type 1a        | 5479   | +113.67          | Regulation of cholesterol transport | Hedgehog receptor activity |
| nvd            | Neverland                     | 3759   | +194.61          | Ec dysone biosynthetic process | Oxidoreductase activity            |
| CG15919        |                                | 3706   | +3370.16         | Proteolysis | Metallo carboxypeptidase activity |
| CG4408         |                                | 3221   | +123.83          | Noppera-bo | Glutathione transferase activity |
| CG6310         | Noppera-bo                    | 1663   | +147.05          | Ec dysone biosynthetic process | Monooxygenase activity            |
| Npc1a          | Cytochrome p450 6g2           | 992    | +145.16          | Oxidation-reduction process | Proteolysis |
| dib            | Disembodied                   | 918    | +206.62          | Ec dysone biosynthetic process | Ec dysone 22-hydroxylase activity |
| CG10337        |                                | 792    | +190.80          | Juvenile hormone biosynthetic process | Juvenile hormone acid methyltransferase activity |
| CG9198         |                                | 598    | +152.41          | Juvenile hormone biosynthetic process | Transporter activity |
| jhamt          | Juvenile hormone acid methyltransferase | 587 | +407.92 | Multicellular organism reproduction | Lysozyme activity |
| CG4822         |                                | 534    | +122.62          | Transporter activity | Lysozyme activity |
| CG6426         |                                | 524    | +130.14          | Metabolic process | Oxidoreductase activity |
| CG13101        |                                | 430    | +202.16          | Metabolic process | Galactose binding |
| Tsp42El        | Tetraspanin 42El              | 411    | +131.97          | Oxidoreductase activity | Hedgehog receptor activity |
| Lectin-galC1   | Galactose-specific C-type lectin | 392 | +107.46 | Induction of bacterial agglutination | Protein tyrosine kinase activity |
| tor            | Torso                         | 162    | +101.70          | Metamorphosis | Transferase activity |
| CG30471        |                                | 120    | +264.62          | Cell adhesion | Cell adhesion |
| CG40006        |                                | 111    | +235.07          | Defense response to bacterium | Oxidoreductase activity |
| Cyp6a13        | Cytochrome p450 6a13          | 107    | +509.06          | Cell adhesion | Cell adhesion |

We have selected GO terms that were most informative for our study, other GO terms for each gene can be found at FlyBase (St Pierre et al. 2014).

*C Only Cel RG data are provided, for A14 data see Table S7.

*Regular text = based on experimental evidence, italics = based on predictions or assertions.
spook was expected, given that this enzyme is required only during embryonic ecdysteroidogenesis, and not during larval stages (Ono et al. 2006). Low expression of shade is consistent with ecdysone being activated to 20E in peripheral tissues, and not the PG (Petryk et al. 2003). Multiple genes involved in cholesterol homeostasis were highly expressed. Cholesterol is a critical precursor for synthesis of many hormones (reviewed in Edwards and Ericsson (1999)), and the enhanced expression of Npc1a and Start1 suggests that these proteins are likely the primary ER transporters responsible for cholesterol availability in the PG cells.

The enhanced expression of the prothoracicotropic hormone (PTTH) receptor, torso, is consistent with PTTH being the primary tropic regulator of ecdysteroidogenesis (McBrayer et al. 2007; Rewitz et al. 2009). In the tobacco hornworm Manduca sexta, it is clear that at least two pathways act downstream of PTTH; the Ras-Raf-ERK pathway is dominant during larval development, then, at metamorphosis, a Ca2+ and cAMP-dependent pathway becomes dominant (Rybczynski and Gilbert 2003) (see Figure 2). Until now, little has been noted about the Ca2+- and cAMP-dependent pathway in D. melanogaster, aside from Ca2+ influx

### Table 2 Expression of select genes involved in ecdysteroidogenesis

| Flybase Symbol | Gene Name | FPKM | Fold Enrichment | q-Value |
|----------------|-----------|------|-----------------|---------|
| nobo           | Noppera-bo| 1203 | +126.97         | <0.001  |
| nvd            | Neverland | 3759 | +194.61         | <0.001  |
| spo            | Spook     | 0.6  | +15.73          | 0.3     |
| spok           | Spookier  | 0.0  | 0               | 1       |
| sro            | Shroud    | 775  | +59.80          | <0.001  |
| phm            | Phantom   | 13,305 | +113.35     | <0.001  |
| dib            | Disembodied| 918 | +206.62         | <0.001  |
| sad            | Shadow    | 12,617 | +161.98     | <0.001  |
| shd            | Shade     | 0.8  | +2.68           | 0.09    |
|                 |           |      |                 |         |
| Cholesterol homeostasis |               |      |                 |         |
| Npc1a          | Niemann Pick C type 1a | 5479 | +113.67        | <0.001  |
| Npc2a          | Niemann Pick C type 2a | 139  | +1.01           | 0.006   |
| Start1         | Start1    | 2277 | +90.48         | <0.001  |
| mdy            | Midway    | 129  | +26.03         | <0.001  |
|                 |           |      |                 |         |
| PTTH signaling |             |      |                 |         |
| tor            | Torso     | 162  | +101.70         | <0.001  |
| Ras            | Ras       | 97   | +1.95           | 0.006   |
| Raf            | Raf       | 11   | -2.11           | <0.001  |
| ERK            | ERK       | 0.0  | 0               | 1       |
| Cam            | Calmodulin| 1240 | +2.18          | <0.001  |
| rut            | rutabega  | 18   | -3.16          | <0.001  |
| PKA            | Protein kinase A | 71 | -1.90        | <0.001  |
| RpS6           | Ribosomal protein S6 | 2528 | +1.33       | 0.05    |
| Hr4            | Hormone receptor 4 | 14 | -1.35         | 0.02    |
| Insulin signaling |               |      |                 |         |
| lnR            | Insulin receptor | 12 | -1.49         | <0.001  |
| P3K            | Phosphotidylinositol 3 kinase | 22 | +1.18       | 0.3     |
| Akt            | Akt       | 50   | +1.42          | 0.003   |
| Activin signaling |             |      |                 |         |
| babo           | Baboon    | 47   | -1.13          | 0.4     |
| put            | Punt      | 67   | +3.04          | <0.001  |
| smad2/smox     | Smad on X | 40   | -2.69          | <0.001  |
| Nitric oxide signaling |       |      |                 |         |
| E75            | Ecdysone-induced protein 75 | 54  | -4.18         | <0.001  |
| Hr46           | Hormone receptor-like 46 | 26 | +3.07       | <0.001  |
| ftz-f1         | ftz transcription factor 1 | 2 | -1.97     | 0.002   |
| TOR signaling  |             |      |                 |         |
| TSC1           | TSC1      | 20   | -1.33          | 0.03    |
| TSC2/gig       | TSC2      | 17   | +1.48          | 0.02    |
| Tor            | Target of rapamycin | 23 | +1.04       | 0.8     |
| Serotonin signaling |        |      |                 |         |
| 5-HT1A         | 5-hydroxytryptamine (serotonin) receptor 1A | 1 | -5.70       | <0.001  |
| JH signaling   |             |      |                 |         |
| Met            | Methoprene-tolerant | 4 | -2.90        | <0.001  |
| gce            | Germ cell-expressed bHLH-PAS | 4 | -2.23     | <0.001  |
| 20E signaling  |             |      |                 |         |
| EcR            | Ecdysone receptor | 80 | +1.59       | <0.001  |
| usp            | Ultraspiracle | 28 | -1.93       | 0.01    |

- Only Cel RG data are provided, for A14 data see Table S8.
- Genes located in heterochromatic regions were not included in reference genome. Reads corresponding to these genes were therefore not aligned by Tophat, hence the 0.0 FPKM score.
appearing to be required for ecdysteroidogenesis in dissected D. melanogaster RGs (Henrich 1995). Here, the highly enriched expression of Calmodulin and Rps6 suggests that the Ca²⁺- and cAMP-dependent branch of the PTTH pathway may be conserved in the dipteran lineage (Marchal et al. 2010; Lin et al. 2011). Another possible role for calcium signaling is regulating vesicle-mediated ecdysone release from the PG (Yamanaka et al. 2015). The Ca²⁺ channel/s that facilitate these two calcium-dependent pathways are yet to be identified (Fellner et al. 2005; Marchal et al. 2010), and we have detected at least nine transmembrane calcium transporters (PMCA, Ca-a1T, pain, Prestin, Itp-r83A, Cac, Ca-a1D, Ca-b, and trp) in the transcriptome.

All known key members of the insulin (Colombani et al. 2005; Caldwell et al. 2005; Mirth et al. 2005), activin (Gibbens et al. 2011), nitric oxide (Caceres et al. 2005), TOR (Layalle et al. 2008), and serotonin (Shimada-Niwa and Niwa 2013) pathways were also detected. The JH receptors Met and gce (Jindra et al. 2015) were both present, supporting evidence that JH negatively regulates ecdysone and JH synthesis at the RG (Richard and Gilbert 1991). Both components of the ecdysone receptor heterodimer, Ecr and usp, were also expressed, adding to evidence that 20E is involved in feedback loops in the RG (Koelle et al. 1991; Karim and Thummel 1992; Song et al. 2003; Moeller et al. 2013).

**Uncharacterized cytochrome P450s are enriched in the ring gland**

CYPs play an important role in the RG tissues, with the most well-known being the Halloween genes involved in ecdysteroidogenesis in the PG (Chavez et al. 2000; Warren et al. 2002; 2004; Ono et al. 2006). All CYPs were extracted from the dataset, and expression levels investigated to identify candidate CYPs that may belong in the Black Box, or be involved in sterol modification. CYPs that were significantly enriched in both RG samples are listed in Table 3 and Table S9. Given that developmental CYPs tend to be more highly conserved and phylogenetically stable than those involved in metabolism, the clade stability for each gene across the phylogeny of 12 Drosophila species was noted (Good et al. 2014). Any published RNAi lethality phenotypes (Chung et al. 2009; Guittard et al. 2011; Qiu et al. 2012) were also considered.

The most highly expressed CYPs were the known Halloween genes, plus the CA-specific Cyp6g2, which may be involved in JH synthesis (Chung et al. 2009; Wen et al. 2015). These genes all had expression levels >900 FPKM, but no other CYPs had expression levels in this range. Nonetheless, there were some CYPs with relevant features. Cyp4d1 knockdown is lethal at the pupal stage (Chung et al. 2009; Qiu et al. 2012), and its closest homolog, Bombyx mori Cyp4d25, is induced by PTTH in the PG (Niwa et al. 2011). Cyp4d2 knockdown is also lethal at the pupal stage (Chung et al. 2009). A notable exception from the enriched CYPs is Cyp6t3. Loss of Cyp6t3 was previously shown to disrupt ec dysone biosynthesis (Ou et al. 2011); however, Cyp6t3 transcripts were effectively absent from our RG samples (FPKM > 1).

**Table 3 Ring gland-enriched cytochrome p450 genes, sorted by FPKM value**

| Cytochrome p450 | FPKM | Fold Enrichment | q Value | Annotated Biological Process | Ubiquitous RNAi Knockdown | Clade Stability Across Drosophila Species |
|-----------------|------|-----------------|---------|-----------------------------|--------------------------|----------------------------------------|
| phm             | 13,305 | +113.35 | <0.001 | Ecdysone biosynthetic process | Lethal | Stable |
| sad             | 12,617 | +161.98 | <0.001 | Ecdysone biosynthetic process | n/a | Stable |
| Cyp6g2          | 992   | +145.01 | <0.001 | Oxidation-reduction process | Lethal | Stable |
| dib             | 918   | +206.62 | <0.001 | Ecdysone biosynthetic process | n/a | Stable |
| Cyp6a13         | 107   | +509.06 | 0.007  | Defense response to bacterium | Viable | Gene loss |
| Cyp6v1          | 96    | +8.42   | <0.001 | Oxidation-reduction process | n/a | Stable |
| Cyp12e1         | 70    | +11.96  | <0.001 | Oxidation-reduction process | Viable | Gene gain |
| Cyp310a1        | 52    | +77.94  | 0.002  | Negative regulation of Wnt signaling pathway | n/a | Gene loss |
| Cyp6u1          | 37    | +3.57   | <0.001 | Oxidation-reduction process | n/a | Stable |
| Cyp92           | 20    | +1.84   | <0.001 | Wing disc development | Viable | Gene gain |
| Cyp4g1          | 19    | +2.72   | 0.08   | Lipid metabolic process | Lethal | Stable |
| Cyp303a1        | 15    | +17.58  | <0.001 | Sensory organ development | n/a | Stable |
| Cyp4d2          | 15    | +3.68   | <0.001 | Oxidation-reduction process | Lethal | Gene loss |
| Cyp6d4          | 13    | +2.36   | <0.001 | Wing disc development | Viable | Gene gain |
| Cyp18a1         | 6     | +28.61  | <0.001 | Ecdysteroid catabolic process | Lethal | Stable |

We selected GO terms that were most informative for our study, other GO terms for each gene can be found at FlyBase (St Pierre et al. 2014).

a OnlyCel RG data are provided, for A14 data see Table S9.

b Regular text = based on experimental evidence, italics = based on predictions or assertions.

c Chung et al. (2009), Guittard et al. (2011), and Qiu et al. 2012.

d Good et al. (2014).

**RNAi knockdown of ring gland-enriched cytochrome p450s:** To establish whether any of the RG-enriched CYPs play an important role in the RG, we investigated their ubiquitous and RG-specific RNAi knockdown viability. A subset of the enriched CYPs (Cyp4g1, Cyp4d2, Cyp6a1, and Cyp6v1) was tested based on expression level, previously reported RNAi lethality (Chung et al. 2009; Guittard et al. 2011; Qiu et al. 2012), and/or the stability of their gene clade (Good et al. 2014).

For Cyp4g1, 100% pupal lethality was observed for ubiquitous knockdown, as previously reported by Chung et al. (2009) and Qiu et al. (2012) (n > 250) (see Figure 4, A and B). This is consistent with the 100% pupal lethality observed by Chung et al. (2009), and with the EMS-induced K350X mutation that causes lethality (Haelterman et al. 2014). The 4% of individuals that survived to adulthood all had asymmetrical melanization on their wings (see Figure 4D). This phenotype is reminiscent of the wing patterning of Drosophila suzukii, and motivated us to look for this gene in the D. suzukii genome sequence. Interestingly, Cyp4d2 is missing from the current D. suzukii genome assembly, although a short
The absence of incidence, may be present in the genome but missing in the assembly stretch of missing bases provides the possibility that the gene, by coincidence, may be present in the genome but missing in the assembly (see Figure S4). The absence of Cyp4d2 in the D. suzukii transcriptome datasets (male and female) in which genes such as the Halloween CYPs are present, adds support to the proposition that Cyp4d2 has genuinely been lost in D. suzukii. Wing spots in the Oriental species of the melanogaster species group have been gained and lost multiple times (Kopp and True 2002), and multiple loci determine their presence and size (Yeh and True 2014). D. biarmes, a species closely related to D. suzukii, has wing spots, and its genome does contain intact Cyp4d2, in each of the RG subtissues had no effect on viability (see Figure 4A and Figure S3).

Figure 4 RNAi knockdown of RG-enriched cytochrome p450s. (A) Cytochrome p450s were knocked down ubiquitously (tubulin-GAL4), and with a PG-specific driver (5’phm-GAL4), and the resulting progeny scored for viability (n > 250). Each bar represents mean ± SEM. Significance was calculated using a Student’s t-test (*** P < 0.0001). (B, C) Representative pupae and larvae at the time of lethality and equivalent wild-type individuals. Ubiquitous knockdown is shown for Cyp4g1 and Cyp4d2. PG-specific knockdown is shown for Cyp6u1. (D) Adults that survived ubiquitous Cyp4d2 knockdown had variable, asymmetrical melanization on their wings (arrows). (E) qPCR reveals significantly lowered levels of Kr-h1, a juvenile hormone primary response gene, and Cyp6g2 transcripts in Cyp6g2 knockdown larvae. Significance was calculated using a Student’s t-test (*** P < 0.0001). (F) Quantity of ecdysteroids in the hemolymph and RG-CNS complexes of wandering third instar larvae is severely reduced in Cyp6u1 PG-RNAi larvae. Bars represent the mean ± SEM of three independent samples. Significance was calculated using a Student’s t-test (** P < 0.05).

For Cyp6u1, ubiquitous knockdown was 100% lethal (see Figure 4A) (n > 250), with most lethality occurring at the first larval instar (32%), second larval instar (10%), and third larval instar (54%) (n = 50) (see Figure 5A). Larvae often died during or shortly after molting. PG-specific knockdown of Cyp6u1 was 92% lethal (see Figure 4, A–C), with lethality occurring at the first larval instar (18%), second larval instar (16%), third larval instar (26%), pupation (4%), and eclosion (34%) (n = 50) (see Figure 5B). Once again, larval lethality was often associated with incomplete molting. This result is the first reported evidence that Cyp6u1 may play a critical developmental role in the PG. We also quantified ecdysteroid levels in the hemolymph and RG-CNS complexes of wandering third instar Cyp6u1 PG-specific RNAi larvae. In both the hemolymph and RG-CNS, Cyp6u1 RNAi larvae had severely reduced ecdysteroid levels compared to the GAL4-only control, and comparable ecdysteroid levels to another ec dysone deficient line (5’phm-GAL4 > UAS-EGFP-ban.C; Boulan et al. 2013) (see Figure 4F). These low ecdysteroid levels, combined with the heterochronic lethality and incomplete molting, provide strong evidence that Cyp6u1 may have a role in ecdysteroidogenesis, possibly in the Black Box. While the known Halloween genes all share a characteristic embryonic lethal phenotype for complete loss-of-function (Revitz et al. 2006; Niwa and Niwa 2014), RNAi knockdown of Halloween genes is less severe. Individuals with PG-specific knockdown of phm, dib, or nobo may die as larvae or pupae (Ou et al. 2011; Enya et al. 2014). Other non-Halloween ecdysteroidogenesis genes also have similar heterochronic lethality upon PG-specific RNAi knockdown (Yoshiyama et al. 2006; Niwa et al. 2011; Ou et al. 2011). So far, all of our evidence suggests that Cyp6u1 is involved in ecdysteroidogenesis, but a null allele will reveal whether complete loss-of-function is
embryonic lethal, and thus whether $Cyp6u1$ achieves the status of Hal-
loween gene.

Tissue-specific knockdown of $Cyp6u1$ in the CA and CC did not
result in any phenotypes (see Figure S3).

For $Cyp6v1$, all ubiquitous and RG-specific knockdowns were viable
($n > 500$) (see Figure S3). qPCR indicates that $Cyp6v1$ expression was
actually increased in the $tubulin$-GAL4; UAS-dsRNA-$Cyp6v1$ strain
relative to the $w^{1118}$; $tubulin$-GAL4 background control (see Figure
S2). Thus, the RNAi knockdown was ineffective, and we are unable
to report a conclusive knockdown phenotype for this gene.

Expression of a JH-regulated gene is decreased by $Cyp6g2$ RNAi:
Previous work has shown that $Cyp6g2$ is the only CYP expressed in the
CA, and that RNAi knockdown of $Cyp6g2$ is pupal lethal (Chung et al.
2009). This makes $Cyp6g2$ a promising candidate for the JH synthesis
pathway. Using qPCR, we observed significantly reduced expression
levels of $Kr-h1$, a JH primary response gene (Minakuchi et al. 2008;
Abdou et al. 2011; He et al. 2014), in $Cyp6g2$ RNAi larvae compared to
GAL4-only controls (see Figure 4E). This suggests a decrease in JH
titers in $Cyp6g2$ RNAi larvae, and strengthens the evidence for $Cyp6g2$’s
involvement in JH synthesis.

Gene ontology enrichment analysis
Throughout the first, second, and most of the third, larval instar stages,
the PG cells have a very well developed smooth endoplasmic reticulum
(ER) (Dai and Gilbert 1991). This is typical of cells involved in steroid
synthesis (De Loof 2008). From the wandering third instar stage, how-
ever, the smooth ER begins to regress, and the rough ER becomes
abundant. This is typical of cells involved in protein secretion, suggest-
ing the PGs may have an additional secretory role in the lead up to
pupation. In this study, we have performed gene ontology enrichment
analysis to uncover any genes that may be involved in any nonsteroido-
genic functions. A subset of 288 transcripts was selected for this
analysis using the following criteria: (1) at least 10-fold enrichment
in both RG samples relative to the CNS, and (2) statistically signi-
cificant enrichment in both RG samples relative to the CNS genes (see
Table S10). With these 288 transcripts, we used DAVID Functional
Annotation Clustering (Huang et al. 2009) to identify three biological
processes: lipid biosynthesis, fatty acid metabolism, and immune re-
sponse (see Table 4).

The lipid biosynthesis cluster (18 genes) is the most significantly
enriched, owing to the abundance of genes involved in ecdysone bio-
synthesis ($phm$, $sad$, $nvd$, and $dib$), cholesterol homeostasis ($Npc1a$,
Table 4 Top ranked biological processes represented by RG-enriched transcripts

| Annotation Cluster | Flybase Symbol | Gene Name |
|--------------------|----------------|-----------|
| Enrichment score: 2.76 | | |
| GO:0008610 - lipid biosynthetic process | phm | Phantom |
| GO:0006694 - steroid biosynthetic process | sad | Shadow |
| GO:0008202 - steroid metabolic process | Npc1a | Niemann-Pick type c 1 a |
| GO:0034754 - cellular hormone metabolic process | nvd | Neverland |
| GO:0042446 - hormone biosynthetic process | Start1 | Start1 |
| GO:0042445 - hormone metabolic process | dib | Disembodied |
| GO:0010817 - regulation of hormone levels | jhant | Juvenile hormone acid methyltransferase |
| GO:0042181 - ketone biosynthetic process | hmas | HMG coenzyme A synthase |
| GO:0045456 - ecdysteroid biosynthetic process | CG8306 | |
| GO:0045455 - ecdysteroid metabolic process | CG8239 | |
| GO:0016125 - sterol metabolic process | mdy | Midway |
| GO:0006697 - ecdysone biosynthetic process | Pgd | Phosphogluconate dehydrogenase |
| GO:0016126 - sterol biosynthetic process | CG10932 | |
| GO:0008205 - ecdysone metabolic process | CG8630 | |
| GO:0019731 - secondary metabolic process | jheh1 | Juvenile hormone epoxide hydrolase |
| | yellow-f | yellow-f |
| | CG17928 | |
| | Cyp18a1 | Cytochrome p450 18a1 |
| Enrichment score: 1.68 | | |
| GO:0006631 - fatty acid metabolic process | CG8306 | |
| GO:0006633 - fatty acid biosynthetic process | CG10932 | |
| GO:0016053 - organic acid biosynthetic process | CG8630 | |
| GO:0046394 - carboxylic acid biosynthetic process | CG3267 | |
| | CG17928 | |
| | tan | tan |
| Enrichment score: 1.55 | | |
| GO:0019730 - antimicrobial humoral response | Thor | Thor |
| GO:0019731 - antibacterial humoral response | He | Hemese |
| GO:0009617 - response to bacterium | Tepl | Thioester-containing protein I |
| GO:0006959 - humoral immune response | CG16799 | |
| GO:006955 - immune response | Drs | Drosomycin |
| GO:0042742 - defense response to bacterium | pirk | poor imd response upon knock-in |
| GO:006952 - defense response | LysS | Lysozyme S |
| | psh | Persephone |
| | Tepl | Thioester-containing protein II |

Gene ontology enrichment analysis was carried out using the Functional Annotation Clustering tool of (DAVID 6.7) (Huang et al. 2009). Clusters with enrichment scores >1.3 (equivalent to nonlog P < 0.05) are shown.

Start1, and mdy), ecdysone inactivation (Cyp18a1), and JH biosynthesis (jhant, hms, CG8239, and jheh1). This cluster also, together with the fatty acid metabolism cluster (six genes), reveals a subset of uncharacterized genes not previously associated with any RG subtissues (CG8306, CG10932, CG8630, CG17928, and CG3267). These uncharacterized genes are bioinformatically predicted to modify fatty acids via branching, desaturation, or elongation. Enrichment of these genes may be associated with energy production or cholesterol storage. Another hypothesis is that the PG may use fatty acid deposits as an indicator of nutritional status to regulate ecdysteroidogenesis (Niwa et al. 2011). As a predicted acetacetyl-CoA thiolase, CG10932 may have a role in the mevalonate pathway upstream of JH biosynthesis in the CA (Bellès et al. 2005).

The immune response cluster (nine genes) was an unexpected finding. This cluster includes genes that actively fight microbial infection, specifically an antifungal peptide (Drs), and two antibacterial peptides (LysS, CG16799), plus genes that regulate the immune response. In D. melanogaster, the immune response is primarily orchestrated by the fat body and the hemocytes [reviewed in Hoffmann (2003)]. The cells of the fat body synthesize and secrete antimicrobial peptides upon activation of the Toll and Imd pathways (Lemaître et al. 1995, 1996). The hemocytes, on the other hand, primarily participate in cellular responses such as phagocytosis, melanization, and encapsulation of parasites (Rizki and Rizki 1984), but are also capable of antimicrobial peptide production (Samakovlis et al. 1990). Based on this transcriptome, it is possible that the RG may be a third contributor to the immune response. The level of enrichment of immune response genes observed here would only be explained by RG expression, as we did not detect sufficient levels of fat body or hemolymph contamination (see Figure S1). In addition, we verified RG expression of a GFP-tagged immune response gene, TepII (see Figure S5) (Nagarkar-Jaiswal et al. 2015). While the D. melanogaster RG has not been previously associated with the immune response, proteomic analysis in the PG of the desert locust Schistocerca gregaria has uncovered a number of proteins involved in defense (Boerjan et al. 2012).

Ou et al. (2016) performed gene ontology enrichment analysis on an array-based RG expression dataset. They too identified “hormone biosynthesis” as a significantly enriched term; however, “immune response” and “fatty acid metabolism” were absent from their results. Inspection of their 208 RG-enriched genes reveals that only 107 were significantly enriched in our RG samples, while 65 were significantly depleted in our RG samples or had similar expression levels to the CNS (see Table S1). We attribute these differences in enrichment, and consequent differences in gene ontology results, to at least two factors;
Imd Pathway

Toll Pathway

Quantitative analysis of RG-enriched genes

Ultrastructural analysis of the PG cells in D. melanogaster has previously revealed a well-developed ER and Golgi, suggesting the PG may have a major role in protein secretion (Dai and Gilbert 1991). To identify genes containing an N-terminal signal peptide, the amino acid sequences of all 288 genes enriched at least 10-fold in the RG were submitted to Signal P (Petersen et al. 2011). Of these genes, 112 received a D-score over 0.45, and their products are therefore predicted to enter the secretory pathway, where they will either be retained at the ER, transported to the plasma membrane, or secreted from the cell (see Table S10).

The most abundant class of signal peptide-containing genes are the serine proteases (22/112 genes; 20%) (see Table 5 and Table S12). Secretion of serine proteases into the hemolymph typically initiates proteolytic cascades that then induce various innate immune responses, including melanization (Tang et al. 2006) and antimicrobial peptide synthesis (Ligoxygakis et al. 2002). A number of these RG-enriched serine proteases are known to be upregulated in response to parasitic, fungal, and bacterial infection (Jon99Fi, Jon25Biii, CG9372, and CG15046, psb) (Shah et al. 2008). There were also 26 uncharacterized genes highlighted by our analysis. CG4408, CG14075, and CG11370 are of particular interest as they are expressed very highly, comparable to the Halloween genes rvd, nobo, and dib (Chavez et al. 2000; Yoshiyama et al. 2006; Enya et al. 2014) (see Table 1). These uncharacterized genes may represent some of the most important secreted products in the RG.

Secretome analysis of RG-enriched genes

RG expression of immune response genes

Table 5 RG-enriched serine proteases, sorted by FPKM value

| Flybase Symbol | Gene Name | FPKM |
|----------------|-----------|------|
| CG4572         | Jonah 99Cii | 535  |
| Jon99Cii       | Jonah 99Cii | 108  |
| CG33465        |           | 64   |
| CG33460        |           | 63   |
| CG9372         |           | 46   |
| CG4259         |           | 39   |
| CG15046        |           | 34   |
| CG10663        |           | 22   |
| CG10232        |           | 21   |
| CG4793         |           | 20   |
| CG4927         |           | 18   |
| CG4386         |           | 18   |
| Jon99Fii       | Jonah 99Fii | 16   |
| CG10764        |           | 15   |
| Jon25Biii      | Jonah 25Biii | 12   |
| CG3355         |           | 11   |
| Jon99Fi        | Jonah 99Fii | 11   |
| CG33225        |           | 11   |
| CG33461        |           | 9    |
| Jon66Cii       | Jonah 66Cii | 7    |
| CG8738         | Persephone | 5    |
| psb            |           | 4    |

Table 6 RG expression of key genes in the immune response pathways

| Flybase Symbol | Gene Name                  | FPKMa | Fold Enrichmenta | q-Value |
|----------------|----------------------------|-------|------------------|---------|
| Toll Pathway   |                            |       |                  |         |
| TI             | Toll                       | 26    | -1.46 (+1.64)    | <0.001  |
| Myd88          | Myd88                      | 15    | +1.74            | <0.001  |
| plf            | Pelle                      | 16    | +1.66            | 0.2     |
| tub            | Tube                       | 27    | -1.19            | 0.2     |
| cact           | Cactus                     | 97    | +1.80            | <0.001  |
| Dif            | Dorsal-related immunity factor | 26    | +2.71            | <0.001  |
| Drs            | Drosomycin                 | 31    | +10.77           | 0.001   |
| Imd Pathway    |                            |       |                  |         |
| PGRP-LC        | Peptidoglycan recognition protein LC | 9    | +5.43            | <0.001  |
| imd            | Immune deficiency          | 23    | +1.86            | 0.5     |
| Fadd           | Fas-associated death domain ortholog | 15    | +3.06            | 0.1     |
| Dredd          | Death related ced-3       | 40    | +3.74            | <0.001  |
| Tak1           | TGF-β activated kinase 1   | 27    | -1.38            | 0.01    |
| key            | Kenny                      | 34    | -1.19            | 0.3     |
| ird5           | Immune response deficient 5 | 16    | +4.85            | <0.001  |
| Rel            | Relish                     | 30    | +2.07            | <0.001  |
| DptB           | Diptericin                 | 3.5   | +9.32            | 0.2     |

aUnless otherwise stated, only Cel RG data are provided. For A14 data see Table S13.
bCel and A14 results were significantly different, therefore A14 data are provided in parentheses.
involved in defense against gram-negative bacteria, and its activation leads to expression of antimicrobial peptides such as diptericin (Lemaître et al. 1995, 1996). The enrichment of these Lmd pathway genes suggests that the RG may also be able to detect, and possibly respond to, gram-negative bacterial infections. The enrichment of these Toll and Imd pathway genes could be explained if the PG is able to use the immune status of the larva as an added level of regulation of ecdysteroidogenesis. Given that infection delays pupation (Olcott et al. 2010), we suggest that the PG may be able to detect an infection and then, potentially via the Toll and/or Lmd pathways, directly or indirectly downregulate Halloween genes to postpone metamorphosis.

Conclusion

This transcriptome has provided a fascinating snapshot of the diversity of developmental signaling occurring in the D. melanogaster third instar RG. We discovered a strong enrichment of gene pathways involved in two processes not previously associated with the D. melanogaster RG; immune response and fatty acid metabolism. We identified a set of enriched CYPs, at least two of which appear to be performing an essential developmental role in the RG. Furthermore, we uncovered a surplus of unnamed genes that are highly enriched, and whose characterization may help complete the ecdysone biosynthesis pathway and may even reveal additional unknown processes in the RG. Much of this transcriptome still remains to be explored. As the first complete D. melanogaster RG transcriptome, we hope this resource will fuel further investigations into the RG, and its broader role in governing insect development.

ACKNOWLEDGMENTS

We thank Thomas W. R. Harrop and Robert T. Good for bioinformatic support, and Hector Sandoval and Hugo Bellen for assistance with ELISA and MiMIC experiments. This work was funded by an Australian Research Council Grant, DP130102415 (P.B.), an Australian Postgraduate Award, Science National Scholarship, Dame Margaret Blackwood Soroptimist Scholarship, and John A. McKenzie/Selby Scientific Foundation Award (D.C.).

LITERATURE CITED

Abdou, M. A., Q. He, D. Wen, O. Zyaan, J. Wang et al., 2011 Drosophila Met and Gce are partially redundant in transducing juvenile hormone action. Insect Biochem. Mol. Biol. 41: 938–945.

Adrian, A. B., and J. M. Comeron, 2013 The Drosophila early ovarian transcriptome provides insight to the molecular causes of recombination rate variation across genomes. BMC Genomics 14: 794.

Baehrcke, E. H., 1996 Ecdysone signaling cascade and regulation of Drosophila metamorphosis. Arch. Insect Biochem. Physiol. 33: 231–244.

Bellès, X., D. Martin, and M.-D. Piulachs, 2005 The mevalonate pathway and the synthesis of juvenile hormone in insects. Annu. Rev. Entomol. 50: 181–199.

Berger, E. M., and E. B. Dubrovsky, 2005 Juvenile hormone molecular actions and interactions during development of Drosophila melanogaster. Vitam. Horm. 73: 175–215.

Bharucha, K. N., P. Tarr, and S. L. Zipursky, 2008 A glucagon-like endocrine pathway in Drosophila modulates both lipid and carbohydrate homeostasis. J. Exp. Biol. 211: 3103–3110.

Boerjan, B., K. Vandenbossche, A. De Loof, and L. Schoofs, 2012 In search for non-steroidogenic functions of the prothoracic glands of the desert locust, Schistocerca gregaria: a peptidomic and proteomic approach. Peptides 34: 57–64.

Boulan, L., D. Martin, and M. Milán, 2013 bantam miRNA promotes systemic growth by connecting insulin signaling and ecdysone production. Curr. Biol. 23: 473–478.

Bowes, M., and H. Rembold, 1987 The titer of juvenile hormone during the pupal and adult stages of the life-cycle of Drosophila melanogaster. Eur. J. Biochem. 164: 709–712.

Bustin, S. A., V. Benes, I. A. Garson, J. Hellemans, J. Huguet et al., 2009 The MIQE Guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55: 611–622.

Caceres, L. A., S. Necakov, C. Schwartz, S. Kimber, I. J. H. Roberts et al., 2011 Nitric oxide coordinates metabolism, growth, and development via the nuclear receptor E75. Genes Dev. 25: 1476–1485.

Caldwell, P. M., W. Walkiewicz, and M. Stern, 2005 Ras activity in the Drosophila prothoracic gland regulates body size and developmental rate via ecdysone release. Curr. Biol. 15: 1785–1795.

Chavez, V., G. Marques, J. Delbecque, K. Kobayashi, M. Hollingsworth et al., 2000 The Drosophila disembodied gene controls late embryonic morphogenesis and codes for a cytochrome P450 enzyme that regulates embryonic ecdysone levels. Development 127: 4115–4126.

Chintapatelli, V. R., J. Wang, and J. A. T. Dow, 2007 Using FlyAtlas to identify better Drosophila melanogaster models of human disease. Nat. Genet. 39: 715–720.

Chung, H., T. Sztal, S. Pasricha, M. Srídhar, P. Batterham et al., 2009 Characterization of Drosophila melanogaster cytochrome P450 genes. Proc. Natl. Acad. Sci. USA 106: 5731–5736.

Colombani, J., L. Bianchini, S. Layalle, E. Pondeville, C. Dauphin-Villemant et al., 2005 Antagonistic actions of ecdysone and insulin determine final size in Drosophila. Science 310: 667–670.

Contrino, S., R. N. Smith, D. Butano, A. Carr, F. Hu et al., 2012 modMine: flexible access to modENCODE data. Nucleic Acids Res. 40: D1082–D1088.

Cunningham, F., M. R. Amode, D. Barrett, K. Beal, K. Billis et al., 2015 Ensembl 2015. Nucleic Acids Res. 43: D662–D669.

Dai, J. D., and L. I. Gilbert, 1991 Metamorphosis of the corpus allatum and degeneration of the prothoracic glands during the larval-pupal-adult transformation of Drosophila melanogaster: a cytophysiological analysis of the ring gland. Dev. Biol. 144: 309–326.

Daines, B., H. Wang, L. Wang, Y. Li, Y. Han et al., 2011 The Drosophila melanogaster transcriptome by paired-end RNA sequencing. Genome Res. 21: 315–324.

De Loof, A., 2008 Ecdysteroids, juvenile hormone and insect neuropeptides: recent successes and remaining major challenges. Gen. Comp. Endocrinol. 155: 3–13.

Dietzl, G., D. Chen, F. Schnorrer, K. C. Su, Y. Barinova et al., 2007 A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. Nature 448: 151–156.

Edwards, P. A., and J. Ericsson, 1999 Sterols and isoprenoids: signaling molecules derived from the cholesterol biosynthetic pathway. Annu. Rev. Biochem. 68: 157–185.

Enya, S., T. Arneku, F. Igarashi, M. Iga, H. Kataoka et al., 2014 A Halloween gene noppora-bo encodes a glutathione S-transferase essential for ecdysteroid biosynthesis via regulating the behaviour of cholesterol in Drosophila. Science 341: 6586.

Fellner, S., R. Rybczynski, and L. Gilbert, 2005 Ca²⁺ signaling in prothoracicotropic hormone-stimulated prothoracic gland cells of Manduca sexta: evidence for mobilization and entry mechanisms. Insect Biochem. Mol. Biol. 35: 263–275.

Gemt, W. M. and D. B. Emmert, 2013 Flybase high throughput expression pattern data. Available at: http://flybase.org/reports/FBrf0221009. html. Accessed: November 1st, 2015.

Gibbens, Y. Y., J. T. Warren, L. I. Gilbert, and M. B. O’Connor, 2011 Neuroendocrine regulation of Drosophila metamorphosis requires TGFbeta/Activin signaling. Development 138: 269–2703.

Good, R. T., L. Gramzow, P. Battlay, T. Sztal, P. Batterham et al., 2014 The molecular evolution of cytochrome P450 genes within and between Drosophila species. Genome Biol. Evol. 6: 1118–1134.

Graveley, B. R., A. N. Brooks, J. W. Carlson, M. O. Duff, J. M. Landolin et al., 2011 The developmental transcriptome of Drosophila melanogaster. Nature 471: 473–479.
Mirth, C., J. W. Truman, and L. M. Riddiford, 2005 The role of the pro-

Lin, J. I., N. C. Mitchell, M. Kalcina, E. Tchoubrieva, M. J. Stewart

Koelle, M., W. Talbot, W. A. Segraves, M. T. Bender, P. Cherbas

Guittard, E., C. Blais, A. Maria, J. P. Parvy, and S. Pasricha

Haelterman, N. A., L. Jiang, Y. Li, V. Bayat, H. Sandoval

Layalle, S., N. Arquier, and P. Léopold, 2008 The TOR pathway couples

Henrich, V. C., 1995 Comparison of ecdysteroid production in

He, Q., D. Wen, Q. Jia, C. Cui, J. Wang

Karim, F., and C. Thummel, 1992 Temporal coordination of regulatory

Jindra, M., Uhlirova, M., Charles, JP., Smykal, V., Hill, R. J., 2015 Genetic

Jiang, Y. L., V. Bayat, H. Sandoval

He, Q., D. Wen, Q. Jia, C. Cui, J. Wang

Lemaitre, B., E. Kromer-Metzger, L. Michaut, E. Nicolas, M. Meister et al., 2015 CYP18A1, a cytochrome P450 enzyme, is essential for ecdysteroid biosynthesis in the prothoracic glands of Bombyx and Drosophila. J. Biol. Chem. 279: 35942–35949.

Niwa, R., T. Sakudoh, T. Matsuya, T. Namiki, S. Kasi et al., 2011 Expressions of the cytochrome P450 monooxygenase gene Cyp9g1 and its homolog in the prothoracic glands of the fruit fly Drosophila melanogaster (Diptera: Drosophilidae) and the silkworm Bombyx mori (Lepidoptera: Bombycidae). Appl. Entomol. Zool. (Ipn.) 46: 533–543.

Ocolc, M. H., M. D. Henkels, K. L. Rosen, F. L. Walker, B. Sneh et al., 2010 Lethality and developmental delay in Drosophila melanogaster larvae after ingestion of selected Pseudomonas fluorescens strains. Plos One 5: e12504.

Ono, H., K. F. Rewitz, T. Shinoda, K. Itoyama, A. Petryk et al., 2006 Spook and Spookier code for stage-specific components of the ecdysone biosynthetic pathway in Diptera. Dev. Biol. 298: 555–570.

Ou, Q., and K. King-Jones, 2013 What goes up must come down: transcription factors have their say in making ecdysone pulses. Curr. Top. Dev. Biol. 103: 35–71.

Ou, Q., A. Magic0, and K. King-Jones, 2011 Nuclear receptor DHR4 controls the timing of steroid hormone pulses during Drosophila development. PLoS Biol. 9: e1001160.

Ou, Q., J. Zeng, N. Y. Amakanaka, C. Brakken-Thal, M. B. O’Connor et al., 2016 The insect prothoracic gland as a model for steroid hormone biosynthesis and regulation. Cell Rep. 16: 247–262.

Perry, T., J. Q. Chan, P. Batterham, G. B. Watson, C. Geng et al., 2012 Effects of mutations in Drosophila nicotinic acetylcholine receptor subunits on sensitivity to insecticides targeting nicotinic acetylcholine receptors. Pestic. Biochem. Physiol. 102: 56–60.

Petersen, T. N., S. Brunak, G. von Hejne, and H. Nielsen, 2011 SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat. Methods 8: 785–786.

Petryk, A., J. Warren, G. Marques, M. Jarcho, L. Gilbert et al., 2003 Shade is the Drosophila P450 enzyme that mediates the hydroxylation of ecdysone to the steroid insect molting hormone 20-hydroxyecdysone. Proc. Natl. Acad. Sci. USA 100: 13773–13778.

Qu, Y., C. Tittiger, C. Wicker-Thomas, G. Le Goff, S. Young et al., 2012 An insect-specific P450 oxidative decarboxylase for cuticular hydrocarbon biosynthesis. Proc. Natl. Acad. Sci. USA 109: 14858–14863.

Rewitz, K. F., R. Rybczynski, J. T. Warren, and L. I. Gilbert, 2006 The Halloween genes code for cytochrome P450 enzymes mediating synthesis of the insect molting hormone. Biochem. Soc. Trans. 34: 1256–1260.

Rewitz, K. F., N. Yamanaka, L. I. Gilbert, and M. B. O’Connor, 2009 The insect neuropeptide PTTH activates receptor tyrosine kinase torso to initiate metamorphosis. Science 326: 1403–1405.

Rewitz, K. F., N. Yamanaka, and M. B. O’Connor, 2013 Developmental checkpoints and feedback circuits time insect maturation. Curr. Top. Dev. Biol. 103: 1–33.

Richard, D. S., and L. I. Gilbert, 1991 Reversible juvenile hormone inhibition of ecdysteroid and juvenile hormone synthesis by the ring gland of Drosophila melanogaster. Experientia 47: 1063–1066.

Riddiford, L. M., 1970 Prevention of metamorphosis by exposure of insect eggs to juvenile hormone analogs. Science 167: 287.

Riddiford, L. M., 1993 Hormones and Drosophila development. pp. 899–939 in The Development of Drosophila. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Rizki, T. M., and R. M. Rizki, 1984 The cellular defense system of Drosophila melanogaster, pp. 579–604 in Insect Ultrastructure. Springer, New York.

Rybczynski, R., and L. Gilbert, 2003 Prothoracicotropic hormone stimulated extracellular signal-regulated kinase (ERK) activity: the changing roles of Ca2+ - and cAMP-dependent mechanisms in the insect prothoracic glands during metamorphosis. Mol. Cell. Endocrinol. 205: 159–168.

Samakovlis, C., D. A. Kimbrell, P. Kyösti, A. Engstrom, and D. Hultmark, 1990 The immune response in Drosophila—pattern of cecropin expression and biological activity. EMBO J. 9: 2969–2976.

Shah, P. K., L. P. Tripathi, L. J. Jensen, M. Gahnim, C. Mason et al., 2008 Enhanced function annotations for Drosophila serine proteases: a case study for systematic annotation of multi-member gene families. Gene 407: 199–215.

Shimada-Niwa, Y., and R. Niwa, 2014 Serotonergic neurons respond to nutrients and regulate the timing of steroid hormone biosynthesis in Drosophila. Nat. Commun. 5: 5778.

Sliter, T. J., B. J. Sodlak, F. C. Baker, and D. A. Schooley, 1987 Juvenile hormone in Drosophila melanogaster. Insect Biochem. 17: 161–165.

Song, Q., X. Sun, and X. Y. Jin, 2003 20E-regulated USP expression and phosphorylation in Drosophila melanogaster. Insect Biochem. Mol. Biol. 33: 1211–1218.

St Pierre, S. E., L. Ponting, R. Stefancsik, and P. McQuilton FlyBase Consortium, 2014 FlyBase 102 - advanced approaches to interrogating FlyBase. Nucleic Acids Res. 42: D780–D788.

Tang, H., Z. Kambris, B. Lemaître, and C. Hashimoto, 2006 Two proteases defining a melanization cascade in the immune system of Drosophila. J. Biol. Chem. 281: 28097–28104.

Thummel, C., 2002 Ecdysone-regulated puff genes 2000. Insect Biochem. Mol. Biol. 32: 113–120.

Trappnell, C., A. Roberts, L. Goff, G. Pertea, D. Kim et al., 2012 Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat. Protoc. 7: 562–578.

Van Hiel, M. B., P. Van Wielendaele, L. Temmerman, S. Van Soest, K. Vuerrincks et al., 2009 Identification and validation of housekeeping genes in brains of the desert locust Schistocerca gregaria under different developmental conditions. BMC Mol. Biol. 10: 56.

Vogt, M., 1943 Zur hormonalen Förderung imaginaler Differenzierungsvorgänge bei Drosophila. Naturwissenschaften 32: 37–39.

Warren, J., A. Petryk, G. Marques, M. Jarcho, J. Parvy et al., 2002 Molecular and biochemical characterization of two P450 enzymes in the ecdysteroidogenic pathway of Drosophila melanogaster. Proc. Natl. Acad. Sci. USA 99: 11043–11048.

Warren, J., A. Petryk, G. Marques, J. Parvy, T. Shinoda et al., 2004 Phantom encodes the 25-hydroxylase of Drosophila melanogaster and Bombyx mori: a P450 enzyme critical in ecdysone biosynthesis. Insect Biochem. Mol. Biol. 34: 991–1010.

Wen, D., C. Rivera-Perez, M. Abdou, Q. Jia, Q. He et al., 2015 Methyl farnesoate plays a dual role in regulating Drosophila metamorphosis. PLoS Genet. 11: e1005038.

Wigglesworth, V. B., 1954 The Physiology of Insect Metamorphosis. Cambridge University Press, Cambridge, UK.

Williams, C. M., 1961 The juvenile hormone. II. Its role in the endocrine control of molting, pupation, and adult development in the Cecropia silkworm. Biol. Bull. 121(3): 572–585.

Yamanaka, N., K. F. Rewitz, and M. B. O’Connor, 2013 Ecdysone control of developmental transitions: lessons from Drosophila research. Annu. Rev. Entomol. 58: 497–516.

Yamanaka, N., G. Marqués, and M. B. O’Connor, 2015 Vesicle-mediated steroid hormone secretion in Drosophila melanogaster. Cell 163: 907–919.

Yeh, S. D., and J. R. True, 2014 The genetic architecture of coordinately evolving male wing pigmentation and courtship behavior in Drosophila elegans and Drosophila gunungcola. G3 (Bethesda) 4: 2079–2093.

Yoshiyama, T., T. Namiki, K. Mitani, H. Kataoka, and R. Niwa, 2006 Neverland is an evolutionally conserved Rieske-domain protein that is essential for ecdysone synthesis and insect growth. Development 133: 2565–2574.

Communicating editor: J. M. Comeron
