Inducible Expression of Several *Drosophila melanogaster* Genes Encoding Juvenile Hormone Binding Proteins by a Plant Diterpene Secondary Metabolite, Methyl Lucidone

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**Simple Summary:** Multiple genes encoding juvenile hormone binding proteins are present in all insect species. However, the variety of juvenile hormones is limited in insects. This suggests other roles for juvenile hormone binding proteins in addition to their role as juvenile hormone transporters. Here, we show that seven *Drosophila melanogaster* juvenile hormone binding protein genes are inducible by methyl lucidone, a plant diterpene secondary metabolite, which functions as a juvenile hormone disruptor both in vitro and in vivo. This suggests that the diversity of juvenile hormone binding protein genes may be related to the presence of diverse plant diterpene secondary metabolites.

**Abstract:** Juvenile hormones prevent molting and metamorphosis in the juvenile stages of insects. There are multiple genes encoding a conserved juvenile hormone binding protein (JHBP) domain in a single insect species. Although some JHBPs have been reported to serve as carriers to release hormones to target tissues, the molecular functions of the other members of the diverse JHBP family of proteins remain unclear. We characterized 16 JHBP genes with conserved JHBP domains in *Drosophila melanogaster*. Among them, seven JHBP genes were induced by feeding the flies with methyl lucidone, a plant diterpene secondary metabolite (PDSM). Induction was also observed upon feeding the juvenile hormone (JH) analog methoprene. Considering that methyl lucidone and methoprene perform opposite functions in JH-mediated regulation, specifically the heterodimeric binding between a JH receptor (JHR) and steroid receptor coactivator (SRC), the induction of these seven JHBP genes is independent of JH-mediated regulation by the JHR/SRC heterodimer. Tissue-specific gene expression profiling through the FlyAtlas 2 database indicated that some JHBP genes are mainly enriched in insect guts and rectal pads, indicating their possible role during food uptake. Hence, we propose that JHBPs are induced by PDSMs and respond to toxic plant molecules ingested during feeding.

**Keywords:** juvenile hormone binding protein (JHBP); juvenile hormone disruptor (JHD); methyl lucidone; plant diterpene secondary metabolite (PDSM)

1. **Introduction**

Some juvenile hormone binding proteins (JHBPs), which are insect-specific, have been shown to bind to the juvenile hormones (JH) and have been proposed to function as JH transporters to target tissues and cells [1–4]. Moreover, JHBP protects JH against degradation by JH esterases [5]. JH biosynthesis occurs in the *Corpora allata* gland, which is
located beneath the brains of insects [6]. JHs are then transported to target tissues and cells to mediate JH-dependent regulation, which affects the development [7], reproduction [8], diapause [9], and polyphenism [10], virtually targeting all tissues. To transport JHs and protect them against degradation, JHBP's must be produced by head tissues or secretory tissues into the hemocoel cavity and be present in circulation.

Although JHBP's have been implicated as JH transporters and protect JHs against degradation, the presence of multiple JHBP's suggests roles other than JH transport. Here, we characterized genes with a conserved JHBP domain in *Drosophila melanogaster* genome release 6 (dm6 by the Berkeley Drosophila Genome Project). In contrast, previous reports indicated the presence of a single juvenile hormone, JHIII, in *D. melanogaster* [11]. More recently, JHIII bisepoxide (JHB3) was also detected in whole-body extracts of adult *D. melanogaster* [12]. The presence of excessive copies of JHBP in the *D. melanogaster* genome compared to the number of JHs may indicate the presence of JHBP's with novel functions other than transporting JHs. Indeed, two proteins in the JHBP family in *D. melanogaster*, Daywake (dyw, JHBP8) and Takeout (to, JHBP9), have different roles from those of JH transporters. Dyw functions in neurons as a day-specific anti-siesta gene with little effect on sleep levels during the night or in the absence of light [13]. The protein takeout is implicated in the circadian control of feeding behavior [14,15] and affects male courtship behavior [16].

Null mutants of the methoprene-tolerant (Met) gene exhibit strong resistance to both JH and the JH analog (JHA) methoprene [17,18]. Therefore, Met has been characterized as the JH receptor (JHR) in *D. melanogaster*. The germ cell-expressed (GCE) gene, which is a Met paralog, was identified as a redundant JHR in *D. melanogaster* [17,19–21]. Other insects, except the lower dipterans, possess a single functional Met gene [22]. Insect JHRs bind to JH with a high affinity and activate the transcription of JH-dependent genes [23–25]. JHR, as with the other members of the bHLH-PAS family of transcription factors, requires other bHLH-PAS proteins for optimal functioning [26]. The bHLH-PAS domain-containing steroid receptor coactivator (SRC; i.e., beta FTZ-F1 interacting SRC, FISC in *Aedes aegypti*, or Taiman in *D. melanogaster*) interacts with JHR as a heterodimeric partner during JH-dependent gene regulation in *A. aegypti* [27], *Tribolium castaneum* [28], and the silkworm *Bombyx mori* [24].

Recently, we found that many plant diterpene secondary metabolites (PDSMs) interfere with the JH-mediated binding of JHR and SRC. Among these JH disruptors (JHDs), methyl lucidone (ML) strongly blocked the larval development of *D. melanogaster*, thereby preventing the formation of pupae and adult fruit flies [29]. In this paper, we report that seven of the 16 *D. melanogaster* JHBP genes were strongly (*p* < 0.01) induced by ML. We also found that four of the seven inducible JHBP genes were expressed mainly in the rectal pad, which suggests that their role may be linked to ingestion, as well as JH transport. Combined with the results revealing that the JHBP genes are inducible by the feeding of ML, we propose that the induction by PDSM activates JHBP's to interact with and possibly counteract potentially harmful plant molecules ingested during feeding.

2. Materials and Methods

2.1. Chemicals

The plant diterpene, methyl lucidone, was isolated from *Lindera erythrocarpa* as described previously [30]. Methoprene was purchased from Sigma-Aldrich (St. Louis, MO, USA), and each reagent was prepared as a stock solution in ethanol.

2.2. Insect Rearing and Feeding

Twenty male and 20 female adult flies were added to individual vials, each containing a 3-g artificial diet mixed with either 0.25% ML (w/v), 0.05% methoprene (w/v), or 0.25% ethanol (w/v), as a control. After 2 d of oviposition, the adult flies were removed from the vials, and the eggs laid were allowed to develop. At this sublethal concentration, ML treatment did not affect their development into adults [29]. After five to six days, the
second instar larvae were collected from each vial, and the total RNA was isolated. The RNA was subjected to quantitative polymerase chain reaction (qPCR) analysis (conducted independently in triplicates). To obtain pupae and adult fruit flies, we continued to incubate the eggs until pupariation or eclosion, respectively. The pupae and adult flies (female or male) were collected from each vial, and the isolated total RNA was subjected to qPCR analysis (conducted independently in duplicates).

2.3. RNA Extraction, Primers, and qPCR Analysis

An RNeasy kit (Qiagen, Hilden, Germany) was used to extract total RNA from second instar larvae, pupae, adult females, and adult males that were fed ethanol (control)-, ML-, and methoprene-supplemented diets. cDNAs were synthesized for qPCR using a Tetro cDNA Synthesis Kit (Bioline, London, UK) and 1 µg RNA, as estimated using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Gene-specific primer pairs listed below were designed for the 16 JHBP genes using Primer 3.

- **JHBP1-F**: 5′-GCTGAAGAACATGGAGGCCTTC-3′
- **JHBP1-R**: 5′-CCAGAACCAAGCTGAGAGCATC-3′
- **JHBP2-F**: 5′-GACAACATCGCCAATGGCAAC-3′
- **JHBP2-R**: 5′-CGGAATCTTGGCGAATATGCG-3′
- **JHBP3-F**: 5′-AAGTTTTGTAAACCAATTG-3′
- **JHBP3-R**: 5′-CGCAAGTTGTGAACACTGCTC-3′
- **JHBP4-F**: 5′-CGTGCAAACGCTCTAATCCG-3′
- **JHBP4-R**: 5′-GTTGTACTTCGCCCGGATCCTA-3′
- **JHBP5-F**: 5′-GAATCGGACTACAGCATTAAGG-3′
- **JHBP5-R**: 5′-CTTGACCTTCACAGTGTTGATC-3′
- **JHBP6-F**: 5′-GTGACTAACCCGCTTAGCAGC-3′
- **JHBP6-R**: 5′-GATTTGGTTTCGGATCGAGCG-3′
- **JHBP7-F**: 5′-GTGACTAACCCGCTTAGCAGC-3′
- **JHBP7-R**: 5′-GCGACACGTTGATGACGCCG-3′
- **JHBP8-F**: 5′-CCTTCTCACTCGTTGGACCC-3′
- **JHBP8-R**: 5′-CGGTAACGTCCAGGTAGGTC-3′
- **JHBP9-F**: 5′-CCATGTCGCTCTCTTCTACAC-3′
- **JHBP9-R**: 5′-CTTTCCCAAGTGCAAACGGG-3′
- **JHBP10-F**: 5′-GATCCGGTGCTGAACGATGTC-3′
- **JHBP10-R**: 5′-CGGTAGTGCAGCAAAGAAGTC-3′
- **JHBP11-F**: 5′-GACAGTTGCCTCCTGAGATCG-3′
- **JHBP11-R**: 5′-CCGACTAACGACGACATCCTC-3′
- **JHBP12-F**: 5′-CTACTTCACAACAGCATCGTG-3′
- **JHBP12-R**: 5′-GAACTTGGTCACCTCGGATCG-3′

qPCR was performed using the RealFAST SYBR kit (Geneer, Daejeon, Korea) in 48-well plates on an Eco Real-Time PCR System (Illumina, San Diego, CA, USA). The following two-step thermal cycler program was used for all runs: 95 °C for 3 min; 40 cycles of 95 °C for 5 s and 60 °C for 20 s; and a final melting curve analysis spanning 95 °C for 15 s, 55 °C for 15 s, and 95 °C for 15 s. The Eco Manager Software (Illumina) was used to validate the amplification efficiency and specificity. RNA extraction and qPCR analysis were performed in triplicate, and the average values of each sample were compared.
2.4. Gene Annotation and Subsequent Data Analyses

The 16 JHBP genes harboring a JHBP domain(s) were annotated from the D. melanogaster reference genome dm6. For the annotation, the following protein databases were used: Pfam [31], PF06585 and SMART [32], and SM000700. Almost all 16 JHBP family proteins contain only one JHBP domain, except for JHBP10 (two JHBP domains annotated from Pfam). The majority of JHBP proteins contained a signal peptide, as detected by the SignalP v4.0 program [33], except for JHBP4 (CG10264) and JHBP10 (CG33680), which contained a transmembrane helix region (detected by TMHMM v2.0 [34]), instead. For tissue-specific expression of JHBP genes, genome-wide tissue-specific gene expression profiles available in the FlyAtlas 2 database [35] were used. FlyAtlas 2 is a repository of tissue-specific gene expression based on genome-wide RNA-seq analyses of D. melanogaster genes in adult males, females, and larvae.

3. Results

3.1. Six among 16 Genes Harboring a JHBP Domain Are Inducible by ML at the Larval Stage

We observed the induction of six of the 16 JHBP genes upon ML treatment in all three biological replicates (Figure 1). The inducible expression of these six genes was also observed upon methoprene treatment (Figure 1). The expression profiles of the other 10 JHBP genes are shown in Figure S1.

![Figure 1](image-url)  
*Figure 1. Six D. melanogaster JHBP genes are inducible by ML and methoprene at the second instar larval stage. The gene expression was evaluated by qPCR with gene-specific primers. All three biologically independent replicates showed the induction of these six JHBP genes, and a representative result is presented. Each result shows the average value of three replicates during the RNA isolation and qPCR steps, and the error bars indicate standard deviation. Statistical significance was determined by a t-test. *, p < 0.01 between control and ML; **, p < 0.01 between control and methoprene.*

3.2. Regulation of Four Larval-Inducible JHBP Genes at the Pupal Stage by ML and Methoprene

We tested the expression of six larval-inducible JHBP genes at the pupal stage after feeding ML or methoprene (Figure 2). Four of the six D. melanogaster larval-inducible JHBP genes were relatively abundantly expressed at the pupal stage, and their expression was significantly affected by both ML and methoprene. Among these, two JHBP genes (JHBP2 and JHBP4) were significantly induced by ML, whereas the expression of the other two genes (JHBP11 and JHBP12) was almost completely repressed in the pupal stage (Figure 2). The expression levels of the other two larval-inducible JHBP genes (JHBP1 and JHBP16) and the other ten JHBP genes at the pupal stage were not affected by ML and/or methoprene (Figure S2).
Figure 2. Inducible or repressed expression of four of the six D. melanogaster larval-inducible JHBP genes at the pupal stage upon ML and methoprene treatment. The gene expression levels were evaluated by qPCR with gene-specific primers. We have shown two figures with different scales of the Y-axis to clarify the expression level of each JHBP gene: in the left figure, JHBP2 with relatively high abundance is depicted; on the right, the expression of the three low-abundant JHBP genes is shown. Two independent biological replicates showed the same results, and a representative result is presented. Each result shows the average value of three replicates during RNA isolation and qPCR steps, and the error bars indicate standard deviation. Statistical significance was determined by a t-test. *, \( p < 0.01 \) between control and ML; **, \( p < 0.01 \) between control and methoprene.

3.3. Stage-Specific Expression of 10 JHBP Genes

Next, we tested the stage-specificity of all 16 JHBP genes (Figure 3), which were not inducible upon ML treatment at the larval stage. Some genes showed relatively strong expression at the adult stage: JHBP9 and JHBP10 in the adult females, JHBP5 in the adult males, and JHBP3, JHBP14, and JHBP15 in both the adult females and males. JHBP7 was highly expressed during the pupal stage. JHBP6, JHBP8, and JHBP15 were expressed at relatively low abundances at all stages tested.

JHBP8 encodes Daywake (dyw), which functions as a day-specific anti-siesta gene in neurons [13]. The strongly inducible expression of the JHBP8 gene by ML at adult stages (Figure 4) suggests an additional role for Daywake in the interaction with ML. We did not observe significant induction of JHBP8 by methoprene.

3.4. Tissue Specificity of JHBP Genes

Although we did not directly test the tissue-specific expression of JHBP genes, we utilized genome-wide tissue-specific gene expression profiles available in the FlyAtlas 2 database [35]. FlyAtlas 2 is a repository of tissue-specific gene expression based on genome-wide RNA-seq analyses of D. melanogaster genes in adult males, females, and larvae. Figure 5 shows the tissue specificity of JHBP1. The FlyAtlas 2 results of other JHBP genes are shown in Figure S3.
Figure 3. Stage-specificity of *D. melanogaster* JHBP genes. The second instar larvae (L), pupae (P), eclosed adult females (F), and eclosed adult males (M) were collected from fruit flies grown on a normal diet and were subjected to RNA isolation. The gene expression was evaluated by real-time PCR with gene-specific primers. We have shown two figures with different scales of the Y-axis to clarify the expression level of each JHBP gene: the upper figure depicts 10 JHBP genes with a relatively high abundance; the lower figure shows the six JHBP genes with a relatively low abundance. Two independent biological experiments showed similar results; representative results are shown in the figure. Each result shows the average value of three replicates during RNA isolation and qPCR steps, and the error bars indicate standard deviation.

Figure 4. The JHBP8 gene, which encodes Daywake, is inducible by ML in adult insects. Gene expression was evaluated by real-time PCR with gene-specific primers. A representative result from two biological replicates is depicted. Each result shows the average value of three replicates during RNA isolation and qPCR steps; error bars indicate standard deviation. Statistical significance was determined by a t-test. *, p < 0.01.
In this study, we characterized seven JHBP genes inducible by ML: six JHBP genes inducible at the larval stage and JHBP8 inducible at the adult stage (summarized in Table 1). The induction of six larval-inducible JHBP genes is independent of the JH-mediated regulation by the JHR/SRC heterodimer since the induction was also observed upon feeding the JHA methoprene. Methoprene activates JH-mediated regulation, specifically facilitating the heterodimeric binding between the JHR and SRC. JH-mediated heterodimeric binding between JHR and SRC activates a downstream cascade of JH-dependent genes [27]. In contrast, ML hinders the JH-mediated formation of JHR/SRC heterodimers. If the expression of these inducible JHBP genes were regulated by the JHR/SRC heterodimer, methoprene and ML would have an opposite function on the expression of the JHBP genes.

Table 1. Characteristic summary of 16 JHBP genes. *, genes harboring the putative transmembrane region instead of the putative signal peptide; **, genes encoding proteins with two JHBP domains. Seven inducible JHBP genes are indicated in red.
Table 1. Cont.

| JHBP | GeneID | Gene Expression (Inducibility by Methyl Lucidone) | Flyatlas 2 Enrichments | Note |
|------|--------|--------------------------------------------------|------------------------|------|
| JHBP5 | CG10407 | Mainly expressed in male adults (Figure 3), no significant induction | Male testis | |
| JHBP6 | CG14457 | only basal expression in all stages (Figure 3), no significant induction | Larval and adult midgut | |
| JHBP7 | CG11852 | Mainly expressed in pupal stage (Figure 3), no significant induction | Larval trachea and hindgut | |
| JHBP8 | CG2650 | Mainly expressed and inducible in adults (Figure 4) | Male rectal pad | Daywake |
| JHBP9 | CG11853 | Mainly expressed in female adults (Figure 3), no significant induction | Ubiquitous | Takeout |
| JHBP10 | CG33680 | Mainly expressed in larvae and female adults (Figure 3), no significant induction | None | *, ** |
| JHBP11 | CG17189 | Inducible in larval stage, but strongly repressed in pupal stage (Figures 1 and 2) | Female rectal pad | |
| JHBP12 | CG13618 | Inducible in larval stage, but strongly repressed in pupal stage (Figures 1 and 2) | Adult crop and rectal pad | |
| JHBP13 | CG3945 | Mainly expressed in adults, no significant induction (Figure 3) | Adult head | |
| JHBP14 | CG5867 | Mainly expressed in larvae and adults, no significant induction (Figure 3) | Ubiquitous | |
| JHBP15 | CG17279 | only basal expression in all stages (Figure 3), no significant induction | Adult head | |
| JHBP16 | CG15497 | Inducible in larval stage (Figure 1) | None | |

The induction of six larval-inducible JHBP genes could be mediated by JH in a JHR/SRC heterodimer-independent manner, such as through the juvenile hormone-activated phospholipase C pathway [36]. However, this pathway is activated by JHIII but not by methoprene, as shown in the paper. Therefore, this pathway may not be involved in the regulation of these JHBP genes at the larval stage, which are induced by both methoprene and ML.

Since our results on ML-inducible JHBP genes may be independent of JH regulation, we propose that these ML-inducible JHBP genes are induced in response to PDSMs. Many plant species contain compounds with JHD activity. In all three plants tested: *L. erythrocarpa*, *Solidago serotina*, and *Pinus densiflora* [30,37], the compounds were shown to be diterpene secondary metabolites. PDSMs disrupt insect development by interfering with JH receptor complex formation. We have previously observed that treatment with PDSMs blocks larval development [29,30,37]. Nonetheless, it is still unclear whether the blocking of larval development by PDSMs depends on JH-mediated regulation. PDSMs are present in plants at high concentrations; therefore, we previously observed that the same percentage
of PDSMs as the crude plant extracts resulted in identical phenotypes blocking larval development [29,30,37]. The uptake of PDSMs during the digestion of plants would be harmful to insects irrespective of their function as a JHD in a JH-dependent manner or as agents blocking larval development in a JH-independent manner. In either scenario, PDSMs exert toxic effects on larval development during plant feeding and need to be detoxified by insect systems. JHBPs may act as response molecules against these PDSMs. Interestingly, two JHBP genes, ML-inducible JHBP4 and non-inducible JHBP10, harbor a transmembrane helix region at the N-terminus instead of a signal peptide (Table 1), indicating that these JHBPs would be unable to function as JHBP transporters. Another remarkable is the finding that four of the seven ML-inducible JHBP genes are mainly expressed in the adult rectal pad (Figures 4 and S1), which is involved in water and solute uptake during food ingestion.

Four ML-inducible JHBP genes at the larval stage, JHBP1, JHBP2, JHBP11, and JHBP12, were relatively abundantly expressed at the pupal stage, and their expression was significantly affected by both ML and methoprene. The expression of two JHBP genes, JHBP1 and JHBP2, were induced by ML at both the larval and pupal stages. In contrast, JHBP11 and JHBP12 gene expressions were repressed at the pupal stage, whereas they remained inducible at the larval stage. Interestingly, one of the major tissues expressing JHBP11 and JHBP12 includes the rectal pad. If JHBP11 and JHBP12 expression are linked to PDSM ingestion, their differential expression between the larval and pupal stages may be associated with their role during the uptake and detoxification of PDSMs. Since fruit flies do not ingest food during pupal development, the genes induced at the larval stage would become unnecessary and would need to be shut down at the pupal stage.

In this study, we elucidated the expression profiles of 16 JHBP genes and linked them to defensive roles against the PDSM, methyl lucidone. Further studies on the direct interaction between JHBPs and PDSM are necessary to prove this unequivocally.

**Supplementary Materials:** The following supporting information can be downloaded at: [https://www.mdpi.com/article/10.3390/insects13050420/s1](https://www.mdpi.com/article/10.3390/insects13050420/s1). Figure S1: Ten D. melanogaster JHBP genes are not-inducible by ML and methoprene at the 2nd instar larval stage. Figure S2: Twelve D. melanogaster JHBP genes are not-inducible/not-repressed by ML and methoprene at the pupal stage. Figure S3: Tissue-specific expression of JHBP2–16 genes shown in the FlyAtlas 2 database.

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**Conflicts of Interest:** The authors have no conflicts of interest to declare.

**References**

1. Hidayat, P.; Goodman, W.G. Juvenile hormone and hemolymph juvenile hormone binding protein titers and their interaction in the hemolymph of fourth stadium Manduca sexta. *Insect Biochem. Mol. Biol.* 1994, 24, 709–715. [CrossRef]
2. Kramer, K.J.; Sanburg, L.L.; Kezdy, F.J.; Law, J.H. The Juvenile Hormone Binding Protein in the Hemolymph of Manduca sexta Johannson (Lepidoptera: Sphingidae). *Proc. Natl. Acad. Sci. USA* 1974, 71, 493–497. [CrossRef] [PubMed]
3. Touhara, K.; Lerro, K.A.; Bonning, B.C.; Hammock, B.D.; Prestwich, G.D. Ligand binding by a recombinant insect juvenile hormone binding protein. *Biochemistry* 1993, 32, 2068–2075. [CrossRef] [PubMed]
4. Trowell, S.C. High affinity juvenile hormone carrier proteins in the haemolymph of insects. *Comp. Biochem. Physiol. Part B Comp. Biochem.* 1992, 103, 795–807. [CrossRef]
5. Touhara, K.; Bonning, B.C.; Hammock, B.D.; Prestwich, G.D. Action of juvenile hormone (JH) esterase on the JH-JH binding protein complex. In vitro model of JH metabolism in a caterpillar. *Insect Biochem. Mol. Biol.* 1995, 25, 727–734. [CrossRef]

6. Nijhout, H.F. *Insect Hormones*; Princeton University Press: Princeton, NJ, USA, 2021.

7. Jindra, M.; Palli, S.R.; Riddiford, L.M. The juvenile hormone signaling pathway in insect development. *Annu. Rev. Entomol.* 2013, 58, 181–204. [CrossRef]

8. Giray, T.; Giovanetti, M.; West-Eberhard, M.J. Juvenile hormone, reproduction, and worker behavior in the neotropical social wasp Polistes canadensis. *Proc. Natl. Acad. Sci. USA* 2005, 102, 3330–3335. [CrossRef]

9. Hahn, D.A.; Denlinger, D.L. Energetics of insect diapause. *Annu. Rev. Entomol.* 2011, 56, 103–121. [CrossRef] [PubMed]

10. Hartfelder, K.; Emlen, D.J. 11—Endocrine Control of Insect Polyphenism. In *Insect Endocrinology*, Gilbert, L.I., Ed.; Academic Press: San Diego, CA, USA, 2012; pp. 464–522.

11. Sliter, T.J.; Sedlak, B.J.; Baker, F.C.; Schooley, D.A. Juvenile hormone in *Insects* 2022.

12. Rivera-Perez, C.; Nouzova, M.; Noriega, F.G. A Quantitative Assay for the Juvenile Hormones and Their Precursors Using Fluorescent Tags. *PLoS ONE* 2012, 7, e43784. [CrossRef]

13. Yang, Y.; Edery, I. Daywake, an Anti-siesta Gene Linked to a Splicing-Based Thermostat from an Adjoining Clock Gene. *Curr. Biol.* 2019, 29, 1728–1734.e4. [CrossRef] [PubMed]

14. Sarov-Blat, L.; So, W.V.; Liu, L.; Rosbash, M. The Drosophila takeout Gene Is a Novel Molecular Link between Circadian Rhythms and Feeding Behavior. *Cell* 2000, 101, 647–656. [CrossRef]

15. So, W.V.; Sarov-Blat, L.; Kotarski, C.K.; McDonald, M.J.; Allada, R.; Rosbash, M. takeout, a novel Drosophila gene under circadian clock transcriptional regulation. * Mol. Cell. Biol.* 2000, 20, 6935–6944. [CrossRef] [PubMed]

16. Dauwalder, B.; Tsujimoto, S.; Moss, J.; Mattox, W. The Drosophila takeout gene is regulated by the somatic sex-determination pathway and affects male courtship behavior. *Genes Dev.* 2002, 16, 2879–2892. [CrossRef] [PubMed]

17. Jindra, M.; Uhlirova, M.; Charles, J.P.; Smykal, V.; Hill, R.J. Genetic Evidence for Function of the bHLH-PAS Protein Gee/Met as a Juvenile Hormone Receptor. *PLoS Genet.* 2015, 11, e1005394. [CrossRef]

18. Wilson, T.G.; Ashok, M. Insecticide resistance resulting from an absence of target-site gene product. *Proc. Natl. Acad. Sci. USA* 1998, 95, 14040–14044. [CrossRef] [PubMed]

19. Abdou, M.A.; He, Q.; Wen, D.; Zyaan, O.; Wang, J.; Xu, J.; Baumann, A.A.; Joseph, J.; Wilson, T.G.; Li, S.; et al. Drosophila Met and Gce are partially redundant in transducing juvenile hormone action. *Insect Biochem. Mol. Biol.* 2011, 41, 938–945. [CrossRef]

20. Godlewski, J.; Wang, S.; Wilson, T.G. Interaction of bHLH-PAS proteins involved in juvenile hormone reception in Drosophila. *Biochem. Biophys. Res. Commun.* 2006, 342, 1305–1311. [CrossRef]

21. Liu, Y.; Sheng, Z.; Liu, H.; Wen, D.; He, Q.; Wang, S.; Shao, W.; Jiang, R.J.; An, S.; Sun, Y.; et al. Juvenile hormone counteracts the bHLH-PAS transcription factors MET and GCE to prevent caspase-dependent programmed cell death in Drosophila. *Development* 2009, 136, 2015–2025. [CrossRef]

22. Wang, S.; Baumann, A.; Wilson, T.G. *Drosophila melanogaster* Methoprene-tolerant (Met) gene homologs from three mosquito species: Members of PAS transcriptional factor family. *J. Insect Physiol.* 2007, 53, 246–253. [CrossRef] [PubMed]

23. Charles, J.P.; Iwema, T.; Epn, V.C.; Takaki, K.; Rynes, J.; Jindra, M. Ligand-binding properties of a juvenile hormone receptor, Methoprene-tolerant. *Proc. Natl. Acad. Sci. USA* 2011, 108, 21128–21133. [CrossRef] [PubMed]

24. Kayukawa, T.; Minakuchi, C.; Namiki, T.; Togawa, T.; Yoshiyama, M.; Kamimura, M.; Mita, K.; Imanishi, S.; Kiuchi, M.; Ishikawa, Y.; et al. Transcriptional regulation of juvenile hormone-mediated induction of Kruppel homolog 1, a repressor of insect metamorphosis. *Proc. Natl. Acad. Sci. USA* 2012, 109, 11729–11734. [CrossRef] [PubMed]

25. Miura, K.; Oda, M.; Makita, S.; Chinzei, Y. Characterization of the Drosophila Methoprene-tolerant gene product. *Journal of the Entomological Society* 1995, 58, 95, 136, 139, 29.

26. Kewley, R.J.; Whitelaw, M.L.; Chapman-Smith, A. The mammalian basic helix-loop-helix/PAS family of transcriptional regulators. *PLoS Genet.* 2005, 29, 727–734. [CrossRef]

27. Kewley, R.J.; Whitelaw, M.L.; Chapman-Smith, A. The mammalian basic helix-loop-helix/PAS family of transcriptional regulators. *Int. J. Biochem. Cell Biol.* 2004, 36, 189–204. [CrossRef]

28. Li, M.; Mead, E.A.; Zhu, J. Heterodimer of two bHLH-PAS proteins mediates juvenile hormone-induced gene expression. *Proc. Natl. Acad. Sci. USA* 2011, 108, 638–643. [CrossRef] [PubMed]

29. Zhang, Z.; Xu, J.; Sheng, Z.; Sui, Y.; Palli, S.R. Steroid receptor co-activator is required for juvenile hormone signal transduction through a bHLH-PAS transcription factor, methoprene tolerant. *J. Biol. Chem.* 2011, 286, 8437–8447. [CrossRef]

30. Shin, S.W.; Jeon, J.H.; Jeong, S.A.; Kim, J.A.; Park, D.S.; Shin, Y.; Oh, H.W. A plant diterpene co-activates juvenile hormone-mediated gene regulation during Drosophila melanogaster larval development. *PLoS ONE* 2018, 13, e0200706. [CrossRef]

31. Lee, S.H.; Oh, H.W.; Fang, Y.; An, S.B.; Park, D.S.; Song, H.H.; Oh, S.R.; Kim, S.Y.; Kim, S.; Kim, N.; et al. Identification of plant compounds that disrupt the insect juvenile hormone receptor complex. *Proc. Natl. Acad. Sci. USA* 2015, 112, 1733–1738. [CrossRef]

32. Mistry, J.; Chuguransky, S.; Williams, L.; Oureshi, M.; Salazar, G.A.; Sonnhammer, E.L.L.; Tosatto, S.C.E.; Paladin, L.; Raj, S.; Richardson, L.J.; et al. Pfam: The protein families database in 2021. *Nucleic Acids Res.* 2021, 49, D412–D419. [CrossRef]

33. Letunic, I.; Bork, P. 20 years of the SMART protein domain annotation resource. *Nucleic Acids Res.* 2018, 46, D493–D496. [CrossRef]

34. Petersen, T.N.; Brunak, S.; von Heijne, G.; Nielsen, H. SignalP 4.0: Discriminating signal peptides from transmembrane regions. *Nat. Methods* 2011, 8, 785–786. [CrossRef] [PubMed]

35. Krogh, A.; Larsson, B.; von Heijne, G.; Sonnhammer, E.L. Predicting transmembrane protein topology with a hidden Markov model: Application to complete genomes. *J. Mol. Biol.* 2001, 305, 567–580. [CrossRef] [PubMed]
35. Leader, D.P.; Krause, S.A.; Pandit, A.; Davies, S.A.; Dow, J.A.T. FlyAtlas 2: A new version of the *Drosophila melanogaster* expression atlas with RNA-Seq, miRNA-Seq and sex-specific data. *Nucleic Acids Res.* 2018, 46, D809–D815. [CrossRef] [PubMed]

36. Liu, P.; Peng, H.-J.; Zhu, J. Juvenile hormone-activated phospholipase C pathway enhances transcriptional activation by the methoprene-tolerant protein. *Proc. Natl. Acad. Sci. USA* 2015, 112, E1871–E1879. [CrossRef]

37. Oh, H.W.; Yun, C.S.; Jeon, J.H.; Kim, J.A.; Park, D.S.; Ryu, H.W.; Oh, S.R.; Song, H.H.; Shin, Y.; Jung, C.S.; et al. Conifer Diterpene Resin Acids Disrupt Juvenile Hormone-Mediated Endocrine Regulation in the Indian Meal Moth *Plodia interpunctella*. *J. Chem. Ecol.* 2017, 43, 703–711. [CrossRef]