Hormonal regulation of the humoral innate immune response in Drosophila melanogaster

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SUMMARY

Juvenile hormone (JH) and 20-hydroxy-ecdysone (20E) are highly versatile hormones, coordinating development, growth, reproduction and aging in insects. Pulses of 20E provide key signals for initiating developmental and physiological transitions, while JH promotes or inhibits these signals in a stage-specific manner. Previous evidence suggests that JH and 20E might modulate innate immunity, but whether and how these hormones interact to regulate the immune response remains unclear. Here we show that JH and 20E have antagonistic effects on the induction of antimicrobial peptide (AMP) genes in Drosophila melanogaster. 20E pretreatment of Schneider S2* cells promoted the robust induction of AMP genes, following immune stimulation. On the other hand, JH III, and its synthetic analogs (JHa) methoprene and pyriproxyfen, strongly interfered with this 20E-dependent immune potentiation, although these hormones did not inhibit other 20E-induced cellular changes. Similarly, in vivo analyses in adult flies confirmed that JH is a hormonal immuno-suppressor. RNA silencing of either partner of the ecdysone receptor heterodimer (EcR or Usp) in S2* cells prevented the 20E-induced immune potentiation. In contrast, silencing methoprene-tolerant (Met), a candidate JH receptor, did not impair immuno-suppression by JH III and JHa, indicating that in this context MET is not a necessary JH receptor. Our results suggest that 20E and JH play major roles in the regulation of gene expression in response to immune challenge.

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Key words: Drosophila, innate immunity, humoral immune response, antimicrobial peptides, juvenile hormone, ecdysone, hormone receptors.

INTRODUCTION

To defend themselves against infectious pathogens, insects like Drosophila use an innate immune system, a primary defense response evolutionarily conserved among metazoans (Janeway, 1989; Medzhitov and Janeway, 1998; Hoffmann and Reichhart, 2002; Tzou et al., 2002; Hoffmann, 2003). Insects have multiple effector mechanisms to combat microbial pathogens. Infection or wounding stimulates proteolytic cascades in the host, causing blood clotting and activation of a phenoloxidase cascade leading to melanization. Cellular immunity involves hemocytes (blood cells), which mediate phagocytosis, nodulation and encapsulation of pathogens. Systemic and local infections also induce a robust antimicrobial peptide (AMP) response. For example, in a systemic infection, AMPs are rapidly produced in the fat body (the equivalent of the mammalian liver) and secreted into the hemolymph (bloodstream) (Gillespie et al., 1997; Kimbrell and Beutler, 2001; Hoffmann, 2003).

The molecular events initiating the transcriptional induction of AMP genes are well characterized (Silverman and Maniatis, 2001; Tzou et al., 2002; Hoffmann, 2003; Kaneko and Silverman, 2005). Upon infection, Drosophila recognizes pathogens using microbial pattern recognition receptors (PRRs), such as peptidoglycan recognition proteins (PGRPs) and Gram-negative binding proteins (GNBPs). Binding of pathogen-derived molecules to these receptors activates two signaling cascades, the Toll pathway and the immune deficiency (IMD) pathway. While the Toll pathway responds to many Gram-positive bacteria and fungal pathogens and activates the nuclear factor kappa B (NF-κB) transcription factors Dorsal and Dif (Dorsal-related immunity factor), the IMD pathway responds to many Gram-negative bacteria and activates the NF-κB homolog Relish. Subsequently, these NF-κB factors induce the expression of a broad range of AMP genes that are effective against Gram-negative and -positive bacteria (e.g. Attacin, Cecropin, Diptericin) and fungi (e.g. Drosomycin, Metchnikowin) (Engström, 1999; Lehrer and Ganz, 1999; Silverman and Maniatis, 2001; Tzou et al., 2002; Hoffmann, 2003). Because the immune system of insects has much in common with the innate immune response of mammals, Drosophila is an excellent model for studying the mechanisms of innate immunity (Silverman and Maniatis, 2001; Hoffmann and Reichhart, 2002).

Increasing evidence suggests that hormones and nuclear hormone receptors systemically regulate adaptive and innate immunity in vertebrates (Rollins-Smith et al., 1993; Rollins-Smith, 1998; Webster et al., 2002; Glass and Ogawa, 2006; Pascual and Glass, 2006; Chow et al., 2007). In mammals, several nuclear hormone receptors have been implicated in regulating innate immunity and proinflammatory gene expression, including peroxisome-proliferator-activated...
receptors (PPARs), liver X receptors (LXRs), vitamin D receptors (VDRs), estrogen receptors (ERs), and the glucocorticoid receptor (GR) (Ricote et al., 1998; Beagley and Gockel, 2003; Joseph et al., 2003; Smoak and Cidlowski, 2004; Glass and Ogawa, 2006; Ogawa et al., 2005). For example, GR represses proinflammatory NF-kB targets, and VDR and its ligand 1,25-dihydroxyvitamin D3 induce expression of the human AMPs cathelicidin (camp) and defensin β2 (defB2) (Wang et al., 2004; Glass and Ogawa, 2006; Schwab et al., 2007; Chow et al., 2007).

In contrast, little is known about the hormonal regulation of immunity in invertebrates such as insects. Several findings suggest that the steroid hormone 20-hydroxy-ecdysone (20E), an important regulator of development, metamorphosis, reproduction and aging in insects (Nijhout, 1994; Kozlova and Thummel, 2000; Tu et al., 2006), modulates cellular and humoral innate immunity. In the mosquito Anopheles gambiae, 20E induces expression of prophenoloxidase I (PPO1), a gene containing ecdysoid regulatory elements (Ahmed et al., 1999; Müller et al., 1999). In Drosophila melanogaster, 20E causes mbn-2 cells, a tumorous blood cell line, to differentiate into macrophages and to increase their phagocytic activity (Dimarcq et al., 1997), and injection of mid-third instar larvae with 20E increases the phagocytic activity of plasmatocytes (Lanot et al., 2001). 20E signaling is also required for Drosophila lymph gland development and hematopoiesis, both necessary for pathogen encapsulation (Sorrentino et al., 2002), and in flesh fly larvae (Neobelliera bullata), 20E promotes the nodulation reaction (Franssens et al., 2006). In terms of humoral immunity, 20E renders D. melanogaster mbn-2 cells and flies competent to induce AMP genes such as Diptericin (Dpt) and Drosomycin (Drs) (Meister and Richards, 1996; Dimarcq et al., 1997; Silverman et al., 2000). The ability to express Dpt in fly larvae depends on the developmental stage; Dpt expression could be induced by infection only after third instar larvae were mature enough to produce sufficient 20E (Meister and Richards, 1996). 20E also promotes expression of the immunoglobulin hemolin in the fat body of diapausing pupae of the Cecropia moth (Hyalophora cecropia) (Roxström-Lindquist et al., 2005). In contrast, 20E may also counteract immune function, since the Toll ligand dorsal, the Toll effector spätzle, and several AMPs were downregulated at the onset of Drosophila metamorphosis in a 20E-dependent manner in gene profiling studies (Beckstead et al., 2005). Similarly, 20E downregulates antibacterial activity in diapausing larvae of the blowfly (Calliphora vicina) (Chernysh et al., 1995). Thus, 20E might either induce or suppress innate immunity, depending on the developmental stage and immune response assayed.

While pulses of 20E provide signals for initiating developmental and physiological transitions (Kozlova and Thummel, 2000), juvenile hormone (JH) specifically promotes or inhibits 20E signaling in a stage-specific manner (Nijhout, 1994; Riddiford, 1994; Berger and Dubrovsky, 2005; Flatt et al., 2005). Recent results suggest that JH – like 20E – might modulate immunity in insects. In the tobacco hornworm (Manduca sexta), JH inhibits granular phenoloxidase (PO) synthesis and thus prevents cuticular melanization (Hiruma and Riddiford, 1998); likewise, JH reduces PO levels and suppresses encapsulation in the mealworm beetle (Tenebrio molitor) (Rolf and Siva-Jothy, 2002; Rantala et al., 2003). In honeybees (Apis mellifera), JH-mediated downregulation of the yolk precursor vitellogenin reduces hemocyte number (Amdam et al., 2004), and in flesh fly larvae (Neobelliera bullata) JH suppresses the 20E-induced nodulation reaction (Franssens et al., 2006). These findings suggest that 20E is typically a positive regulator of innate immunity, while JH acts as an immuno-suppressor (Flatt et al., 2005). Although JH induces expression of the AMP Ceratotoxin A in female accessory glands of the medfly (Ceratitis capitata), this peptide is not induced by bacterial infection (Manetti et al., 1997). Thus, it remains unclear how JH affects the expression of pathogen-inducible AMPs in humoral immunity. Furthermore, whether and how 20E and JH interact to regulate AMP expression has not been investigated.

Here we demonstrate that 20E promotes humoral immunity by potentiating AMP induction in D. melanogaster, but that this 20E-induced response is specifically and strongly inhibited by JH and juvenile hormone analogs (JHα). We further show that immune induction by 20E requires ecdysone receptor (EcR)/ultraspiracle (USP), but that immune suppression by JH is independent of the putative JH receptor methoprene-tolerant (MET).

**MATERIALS AND METHODS**

**Hormones**

For hormone application in Schneider S2* cells and flies we used the following compounds: 20-hydroxy-ecdysone [20E, (2β,3β,5β,22R)-2,3,14,20,22,25-hexahydroxycholest-7-en-6-one; Sigma, St Louis, MO, USA; 1 mmol l–1 stock solution in water]; juvenile hormone III (JH III ‘methyl epoxy farnesoate’, 10-epoxy-3,7,11-trimethyl-trans-trans-2,6-dodecadienoic acid methyl ester, isolated from Manduca sexta, Sigma, 3.7 mmol l–1 stock in ethanol for cell culture and 187 mmol l–1 stock in acetone for topical treatment of flies); the JH analog (JHa) methoprene [isopropyl-(2E,4E)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienolate, Sigma (PESTANAL, racemic mixture), 7.9 mmol l–1 stock in ethanol]; and the JHa pyriproxyfen [2-[(methyl-2-(4-phenoxyphenoxy)-ethoxy)-pyridine; ChemService, Inc., West Chester, PA, USA; 6.4 mmol l–1 stock in ethanol]. For dose–response experiments, hormones were freshly prepared as stock solutions in ethanol; final dilutions in cell culture were in water with 0.01% ethanol. The JHα methoprene and pyriproxyfen are more soluble, more potent and more resistant to in vivo degradation than JH III; JHα can act as a faithful JH agonist, both in vivo and in vitro (Cherbas et al., 1989; Riddiford and Ashburner, 1991; Wilson, 2004; Zera and Zhao, 2004; Flatt and Kawecki, 2007). For details on hormone delivery, see below.

**Drosophila stocks and culture**

We used the yellow white (y, w) strain for the microarray experiment and the northern blot on Diptericin mRNA (courtesy of Eric Rulifson, University of California, San Francisco); for the green fluorescent protein (GFP) reporter assays, we used the Drosomycin-GFP reporter strain DDI [y; P(ry+, Dpt-lacZ), P(w+, Drs-GFP); cn, bw; courtesy of Dominique Ferrandon, CNRS, Strasbourg] (Reichhart et al., 1992; Ferrandon et al., 1998) and the Diptericin-GFP reporter strain DIG [w; P(Dpt-GFP, w+)D3-2, P(Dpt-GFP, w+)D3-4; courtesy of Bruno Lemaitre, EPFL, Lausanne] (Vodovar et al., 2005). Flies were reared on a standard fly food medium consisting of cornmeal/sugar/yeast/agar at 25°C, 40% relative humidity, and a 12 h light–dark cycle.

**Microarrays**

To examine the transcriptional response of y, w flies to treatment with exogenous JH, we performed a microarray experiment on uninfected females treated with JH or solvent (control). Since the physiological effects of JH are better understood in females than in males, we only used females in this experiment. Flies were grown on regular yeast diet, switched to no-yeast food within 1 h of eclosion, and yeast-starved for 5 days posteclosion to lower their endogenous JH titer and to synchronize their physiology (see Tu and Tatar, 2003;
Gershman et al., 2007). Subsequently, flies were anesthetized on ice and topically treated with 0.1 μl of 187 mmol·l⁻¹ JH III in acetone or with 0.1 μl 100% acetone (control) using a 1 μl Hamilton syringe with a repeating dispenser; 12 h after hormone administration, samples were snap-frozen in liquid nitrogen and stored at −80°C. Total RNA from whole flies was isolated from samples (two JH samples, two control samples, each with 30 females) by lysis, as previously described (Gershman et al., 2007). cDNA products were hybridized at the Brown University Genomics Core Facility to Affymetrix GeneChip Drosophila_1 Genome Arrays (two replicate chips per treatment). The dataset consisted of 14,009 probe sets, with 6142 probe sets annotated. Expression data were analyzed for significant over- or underrepresentation of gene ontology (GO) terms with the web application FatiGO (Al-Shahrour et al., 2004), using a two-fold change criterion. To test whether JH treatment significantly suppresses expression of AMPs, we used Student’s t-tests implemented in JMP IN 5.1 (SAS Institute, Cary, NC, USA) (Sall et al., 2004). The microarray dataset has been deposited in Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) with accession number GSE9001. Results of the microarray experiment were confirmed by analyzing two additional independent microarray experiments: one experiment on JH- and solvent-treated γ, w females, following the time course design of Gershman and colleagues (Gershman et al., 2007); the other experiment with S2* cells treated with solvent, JHa (methoprene), 20E or both 20E and JHa (three replicates each; data not shown).

**Fly GFP reporter experiments**

To test whether the JHa methoprene suppresses AMP expression in vivo we used a whole-fly GFP reporter assay of the DD1 (Dros-GFP) and the DIG (Dpt-GFP) strains, combining hormonal manipulation (JHa application vs control) with manipulation of infection status (unjabbed control; sterile, ethanol-jabbed control; and bacteria jabbed). Each of the 2 × 3 × 2 (JHa; control) × (unjabbed; ethanol jabbed; bacteria jabbed) treatment groups consisted of fifteen 3 day old females (total N = 90 females). Prior to manipulating infection status, flies were exposed for 24 h in vials to vaporized JHa methoprene (10 μl at 7.9 mmol·l⁻¹) or 70% ethanol (control; 10 μl). The next day, flies were lightly anesthetized with moist CO₂ and jabbed at the abdomen intersegment with a fine (0.2 mm diameter) Minuten pin needle (Fisher Scientific, Pittsburgh, PA, USA), dipped in live Gram-negative bacteria (E. coli, strain 1106; bacterial pellets made by centrifugation of a liquid overnight culture in LB growth medium) or in 70% ethanol (sterile jabbed control), or left unjabbed. Twenty-four hours after infection, flies were anesthetized using CO₂ and their GFP expression visualized under fluorescent (FITC) light with a Zeiss Stemi SV11 dissecting scope; images of individual flies were taken with an AxiosCam MRm camera (Carl Zeiss, Jena, Germany; exposure time, 5 s) and processed with AxioVision LE Rel. 4.3 software. For analysis, images were imported into ImageJ (http://rsb.info.nih.gov/ij/). After thresholding images, surface areas of flies were estimated using the polygon selection tool. Image exposure time and threshold parameters were kept constant for all images. Data were analyzed with two-way analysis of variance (ANOVA) implemented in JMP IN 5.1 (Sall et al., 2004), using infection status and hormone treatment as fixed factors.

**S2* cell culture and cell induction**

For cell culture experiments we used an embryonic hemocyte- or macrophage-like Drosophila cell line known as Schneider S2* cells (Samaklovis et al., 1992). S2* cells were maintained at 25°C in Schneider S2 Drosophila medium (Gibco, Gaithersburg, MD, USA; Invitrogen, Carlsbad, CA, USA) or Schneider’s insect media (Sigma), supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, UT, USA), 1% GlutaMax-1 (Invitrogen), and 0.2% Penicillin–Streptomycin (Pen-Strep, Invitrogen). The Diptericin-luciferase cell line (Dpt-luc) was a stable S2* transfectant containing the reporter plasmid pJM648 (Tauszig et al., 2000; Kaneko et al., 2004); at each passage, cells were selected with Geneticin (G418 sulfate, Gibco, Invitrogen, 800 μg·ml⁻¹). Cell counts were made with a Fuchs-Rosenthal ultraplane counting chamber (1/16 mm²; 2/10 mm deep; Hausser Scientific, Horsham, PA, USA). For experiments, cells were immune-stimulated with 1 μg·ml⁻¹ E. coli peptidoglycan (PGN; InvivoGen, San Diego, CA, USA; 1 mg·ml⁻¹ stock) for 5–6 h or left untreated (control). In one experiment, we used crude lipopolysaccharide (LPS) from E. coli (0111:B4; Sigma); the active Drosophila immune-stimulating component of crude LPS has been shown to be PGN (Kaneko et al., 2004). For northern or western blotting without RNA interference (RNAi), cells were plated at 10⁶ cells·ml⁻¹ in six-well tissue culture plates (3 ml of cells per well); after 24 h, cells were split to 10⁵ cells·ml⁻¹ in six-well plates (3 ml cells per well) and incubated with hormones (no hormone; 20E; JH or JHa; JH or JHa plus 20E). For each hormone, we added 3 μl of stock solution per well (see above; 1000× dilution). After 24 h of hormone incubation, cells were stimulated with PGN for 5–6 h or left unstimulated (control). For experiments with Dpt-luc cells, procedures were identical, except that cells were plated at 10⁶ cells·ml⁻¹ in 96-well plates (100 μl cells per well); hormones were administered as 1 μl of stock per well (1000× dilution). Each cell culture experiment was replicated at least four times.

**RNAi**

To study the genetics of the hormonal response we performed RNAi-mediated silencing of Drosophila ecdysone receptor (EcR), ultraspiracle (Usp) and methoprene-tolerant (Met). Double-stranded RNA (dsRNA) was synthesized from a PCR-amplified template, with T7 promoter sequences flanking a ~500 bp fragment of the gene of interest, using the Ribomax kit (Promega, Madison, WI, USA), as previously described (Silverman et al., 2000). dsRNA was purified by phenol/chloroform extraction and ethanol precipitation. As RNAi controls, we used dsRNA for E. coli LacZ (encoding β-galactosidase) or E. coli MalE (encoding maltose binding protein). Primers used to generate dsRNA are described in supplementary material Table S1. dsRNA for MalE was generated using the HiScribe RNAI transcription kit (New England BioLabs, Ipswich, MA, USA); an 808 bp (BglII–EcoRI) fragment of MalE was inserted into the Litmus 28I vector and amplified using the T7 minimal primer. For RNAi-mediated silencing, cells were plated at 10⁶ cells·ml⁻¹ (see above) and then soaked with 30 μg of dsRNA in 1 ml FBS-free medium for 30 min, followed by addition of 2 ml of complete medium. Twenty-four hours later, cells were split to 10⁵ cells·ml⁻¹ in six-well plates (for northern and western blotting) or plated at 10³ cells·ml⁻¹ in 96-well plates (for luciferase assays); subsequently, cells were treated with hormones and immune stimulated, as described above.

**Luciferase reporter assays**

To examine how hormones affect Dpt promoter activity, we performed luciferase assays with Dpt-luc reporter cells in 96-well plates, using 100 μl cells per well (10⁶ cells·ml⁻¹; see above). Five to six hours after induction with PGN, samples on experimental plates were transferred to black 96-well assay plates (BD Falcon, Franklin Lakes, NJ, USA) and lysed for 2 min in Bright-Glo Assay
Re transfected with standard procedures, as previously described (Silverman et al., 2000). Relative (encoding ribosomal protein RP49) was analyzed by RNA blotting reagent (Invitrogen) and expression of or DNA). Total RNA from cultured cells was isolated with TRIzol

For northern blotting, dsRNA, DNA or dsRNA plus DNA were transfected into S2* cells using a standard calcium phosphate method; 24 h after transfection, cells were split, treated with hormones and immune stimulated as described above. After a further 24 h, whole-organism effects of hormonal signaling: hormones can be topically applied or injected, are taken up into the circulation, act on responsive target tissues, and elicit a systemic, whole-organism response (e.g. immune modulation).

**Northern and western blotting**

For northern blotting, dsRNA, DNA or dsRNA plus DNA were transfected into S2* cells using a standard calcium phosphate method. dsRNA and DNA were prepared in 2× BBS [BES-buffered saline; 50 mmol l^{-1} N,N-bis(2-hydroxyethyl)2-aminoethane-sulfonic acid (Sigma), 0.28 mmol l^{-1} NaCl, 1.5 mmol l^{-1} Na_{2}HPO_{4}, at pH 6.95], followed by addition of CaCl_{2}. Transfection mixtures were vortexed thoroughly and, after 15 min of incubation at room temperature, added dropwise to the S2* cells. After 24 h, transfected cells were split, treated with hormone and immune stimulated as described above. As controls we used untransfected and mock-transfected S2* cells (transfected with the transfection mixture only, without dsRNA or DNA). Total RNA from cultured cells was isolated with TRIzol reagent (Invitrogen) and expression of Dpt and control Rp49 (encoding ribosomal protein RP49) was analyzed by RNA blotting as previously described (Silverman et al., 2000). Relative quantification of Dpt expression was performed by comparing the intensities of the experimental bands and the Rp49 control bands. For the northern blot on y, w flies for Dpt mRNA, we followed standard procedures, as previously described (Silverman et al., 2000).

For western blot analysis of USP, cell lysates from S2* cells transfected with Usp RNAi were prepared and 50 μg of protein per lane was applied on a 10% SDS-PAGE gel. After electrophoresis, proteins were transferred onto a PVDF membrane. Non-specific binding was blocked with TBS (25 mmol l^{-1} Tris-HCl, 0.5 mmol l^{-1} NaCl, pH 7.5), supplemented with 5% non-fat dried milk for 1 h at room temperature. Blots were incubated for 2 h at room temperature with a 1:100 dilution of the mouse monoclonal antibody AB11 (courtesy of Carl Thummel, University of Utah School of Medicine) directed against USP (Christianson et al., 1992). After 3×15 min washes in TBST (TBS containing 0.1% Triton X-100), blots were incubated for 1 h in peroxidase-conjugated anti-mouse IgG (Amersham, Little Chalfont, Bucks, UK) diluted 1:2500 in TBS, and washed three times for 15 min with TBST. Proteins were visualized with West Pico SuperSignal (Pierce, Rockford, IL, USA).

MET protein was examined with Western blotting performed on S2* cells transfected with Met dsRNA, Met plasmid expression vector, or double transfected with Met dsRNA and Met expression vector. Transfection with Met expression vector [pAC5.1(C) MET-V5-6×His; estimated molecular mass 82.2 kDa; courtesy of Thomas G. Wilson, Ohio State University] was used because endogenous MET levels in S2* cells were low (data not shown). Transfection was performed using a standard calcium phosphate method; 24 h after transfection, cells were split, treated with hormones and immune stimulated as described above. After a further 24 h, whole-cell extracts were prepared with lysis buffer and 50 μg of protein extract per lane was applied on an 8% SDS-PAGE gel. After electrophoresis, proteins were transferred onto a PVDF membrane and non-specific binding was blocked with TBS containing 0.1% Tween 20 (TBST), supplemented with 10% non-fat dried milk overnight at room temperature. The next day, blots were incubated for 3 h at room temperature with rabbit polyclonal MET antibody (courtesy of Thomas G. Wilson) (Pursley et al., 2000), at a dilution of 1:2500 in 10% milk in TBS, followed by 3×15 min washes in TBST. Blots were incubated for 1 h at room temperature in peroxidase-conjugated anti-rabbit IgG (BioRad, Hercules, CA, USA) diluted at 1:10,000 in 10% milk in TBS and washed three times for 15 min with TBST. Proteins were visualized with West Pico SuperSignal.

**RESULTS**

**Juvenile hormone functions as an immuno-suppressor in vivo**

To examine the transcriptional effects of JH in the fly, we performed a microarray experiment using total RNA from whole bodies of uninfected adult *D. melanogaster* females topically treated with JH III or with solvent (control). FatiGO gene ontology analysis revealed that JH affected the expression of 270 genes at least two-fold, with 110 genes being upregulated and 160 genes downregulated. Remarkably, among the 270 genes regulated by JH, 35 (13.04%) were annotated as genes involved in the response to biotic stimuli such as bacteria, fungi, oxidative stress and starvation (GO:0009607; supplementary material TableS2). These genes were significantly overrepresented in JH-treated flies relative to chance expectation (observed, 13.04%; expected, 7.55%; Fisher’s exact test, *P*=0.0051). Within this GO category, genes responsive to pests, pathogens and parasites (GO:0051707) were significantly enriched (observed, 3.91%; expected, 1.25%; Fisher’s exact test, *P*=0.004). Among the 160 genes downregulated by JH, 17.65% (28 genes) were genes responsive to biotic stimuli, whereas among the 110 genes upregulated by JH only 6.32% (seven genes) belonged to this category. The difference between these percentages was significant (Fisher’s exact test, *P*=0.0159), suggesting that the majority of the 35 biotic response genes regulated by JH are suppressed rather than induced by JH. In

![Fig. 1. Juvenile hormone III (JH III) suppresses basal antimicrobial peptide (AMP) expression in whole flies. Shown are log2-fold change values (JH/control) for 12 AMP transcripts from microarray analyses performed in duplicate. *P*<0.05 (Student’s t-test). Since the physiological role of JH is not well understood in males, we only used females in this array experiment. We suggest that microarrays might be a particularly useful tool when studying whole-organism effects of hormonal signaling: hormones can be topically applied or injected, are taken up into the circulation, act on responsive target tissues, and elicit a systemic, whole-organism response (e.g. immune modulation).
particular, JH significantly suppressed the basal expression of several antimicrobial peptides more than two-fold (supplementary material Table S2). In a separate analysis, relaxing the two-fold change criterion, we found that 6 out of 12 AMP genes, including Dpt and Drs, were significantly suppressed by JH III treatment (Fig. 1; Student’s t-tests, all P<0.05). Thus, JH suppresses the transcription of immunity genes in vivo, even in the absence of infection (Fig. 1).

To verify these expression data we analyzed two additional, independent microarray experiments, one using y, w females flies, the other S2* cells: in both experiments, JH III or JHa treatment reduced the expression of the majority of AMPs (data not shown).

To confirm that JH/JHa suppresses AMP expression in vivo, we analyzed Drs-GFP reporter expression in DD1 females (Fig. 2) and Dpt-GFP reporter expression in DIG females (data not shown). For both reporters, we observed substantial variation among individuals in GFP expression intensity, both within and among treatments, as well as among replicate experiments. Therefore, to test whether JH/JHa treatment suppresses GFP induction upon infection, we estimated Drs-GFP expression using quantitative image analysis.

Infection with Gram-negative E. coli strongly increased Drs-GFP expression (Fig. 2A,B; two-way ANOVA, $F_{1,7} = 7.09, P=0.03$), while treatment with JHa methoprene significantly reduced expression (Fig. 2C,D; $F_{1,7} = 6.6, P=0.0375$), in both uninfected (Fig. 2C,E) and infected flies (Fig. 2D,E; infection×hormone interaction effect: $F_{1,7} = 0.008, P=0.93$). Qualitatively similar results were obtained in independent repeats of this experiment and in trials using flies infected with Gram-positive M. luteus (data not shown). To further confirm the JH-mediated suppression of AMP induction in vivo we performed northern blotting on y, w females and found that infection-induced Dpt expression was reduced 2-fold in females treated with JHa (methoprene) vapor relative to controls exposed to solvent only (data not shown). Thus, JH/JHa suppresses the expression of genes involved in innate immunity, including several AMPs (Figs 1 and 2; supplementary material Table S2).

**20E and JH antagonistically regulate AMPs in S2* cells**

20E promotes AMP expression when whole insects or insect cells in culture are exposed to bacterial stimuli (e.g. Meister and...
Richards, 1996; Dimarcq et al., 1994; Dimarcq et al., 1997; Silverman et al., 2000). Since JH often counteracts 20E-dependent responses (Riddiford, 1994; Dubrovsky, 2005) and might function as an immune-suppressor [see above (see also Flatt et al., 2005)], we first verified that pretreatment with 20E is required for efficient AMP transcription upon immune challenge and then examined whether JHa could repress this response. To monitor AMP gene induction, we performed northern blotting on RNA extracted from S2* cells, using probes for the AMP genes Dpt, Cecropin and Attacin (Fig. 3). S2* cells not exposed to 20E showed little or no AMP gene induction in response to PGN immune stimulation (Fig. 3A, left lanes). In contrast, S2* cells pretreated with 20E 24 h prior to immune challenge robustly and strongly expressed AMPs upon PGN stimulation (Fig. 3A, middle lanes). Similarly, treatment of immune-stimulated Dpt-luc cells with 20E caused a dramatic increase in Dpt promoter activity (80-fold increase), whereas PGN treatment in the absence of 20E had a markedly smaller effect on activation of the Dpt promoter (five-fold increase; Fig. 3B). Thus, the effects on Dpt mRNA transcript levels, as monitored by northern blotting (Fig. 3A), are also reflected at the level of Dpt promoter activity, as monitored in luciferase assays.

We found that cells treated with both JHAs (methoprene) and 20E did not gain the immune capacity of cells treated with 20E alone (Fig. 3A, right lanes; and Fig. 3B, 17-fold decrease compared with 20E alone), confirming our in vivo observation that JH functions as an immuno-suppressor. JHa in the absence of 20E, on the other hand, only weakly suppressed the immune capacity of PGN-stimulated cells (3.4-fold suppression by JHa compared with no hormone control; Fig. 3B). This suggests that JHa is an antagonist of 20E. Dose–response experiments with 20E alone and with JH (or its synthetic analogs methoprene and pyriproxyfen) in the presence of 20E confirmed that JH and JHa antagonize the 20E response (Fig. 4). Increasing concentrations of 20E upon immune stimulation strongly increased Dpt reporter activity (Fig. 4A), but JH and its synthetic analogs decreased this response in a dose-dependent manner (Fig. 4B, C, D).

To determine the time course of 20E-mediated potentiation, we assayed Dpt reporter activity in response to a range of 20E incubation times. 20E-mediated potentiation of the immune response required at least 18 h of pretreatment with 20E (Fig. 5A; and data not shown). Similarly, to determine the timing of JHa suppression of 20E-mediated potentiation, Dpt reporter activity was assayed across a range of JHa incubation times. Cells were incubated with 20E for a total of 24 h; the time at which JHa was added to cell culture varied among treatments. 20E-induced Dpt reporter activity was rapidly suppressed by JHa within 4 h of JHa exposure; exposure of cells to JHa for more than 4 h did not markedly enhance inhibition of the 20E response (Fig. 5B).

In addition to modulating the immune responsiveness of S2* cells, 20E also induced growth arrest (Fig. 6) and changes in cellular morphology in these cultured cells (data not shown), as previously reported (reviewed in Echalier, 1997). However, the JHa methoprene did not affect these 20E-mediated developmental phenotypes. Cells treated with both 20E and JHa stopped proliferating and morphologically differentiated, just like cells treated with 20E alone (Fig. 6; and data not shown). Thus, JHa appears to be a rapid and specific inhibitor of the ability of 20E to increase immune capacity.

**20E induction of AMPs requires EcR/USP**

20E signaling typically requires binding of the hormone to a heterodimer formed by two nuclear hormone receptor family members, ecdysone receptor (EcR) and ultraspiracle (Usp) (Koelle et al., 1991; Thomas et al., 1993; Yao et al., 1993; Hall and Thummel, 1998). We therefore tested whether 20E potentiation of Dpt requires EcR and Usp (Figs 7 and 8; supplementary material Table S3). RNAi directed against EcR completely prevented 20E-mediated potentiation of Dpt mRNA expression and reporter activity (Fig. 7A and Fig. 8A; supplementary material Table S3). Similarly, RNAi directed against Usp abolished 20E potentiation (Fig. 7B and Fig. 8B; supplementary material Table S3); western blot analysis confirmed the effectiveness of Usp RNAi (Fig. 7C). Although 20E-mediated potentiation of Dpt reporter activity was markedly decreased by RNAi targeting of EcR and Usp, the residual level of reporter activity was still inhibited by JHa (Fig. 8A, B), but this suppression was not statistically significant (supplementary material Table S3; interaction contrasts analysis). Thus, it is difficult to firmly conclude whether or not USP is required for the JHa-mediated suppression of immune inducibility. Similar results were obtained when using JH III (data not shown). However, it is very clear that 20E regulates Dpt expression and promoter activity by signaling through EcR/USP.
In contrast to 20E, the mechanisms underlying signal transduction downstream of JH remain unknown (Wilson, 2004; Berger and Dubrovsky, 2005; Dubrovsky, 2005; Flatt et al., 2005). Therefore, to understand how JH down-modulates immune function, we asked whether repression of the 20E response by JH and JHa depends on Met, a candidate receptor for JH (Wilson and Fabian, 1986; Shemshedini and Wilson, 1990; Shemshedini et al., 1990; Wilson and Ashok, 1998; Pursley et al., 2000). RNAi-mediated silencing of Met did not affect 20E potentiation of Dpt promoter activity and mRNA expression (Fig. 8C and Fig. 9A; supplementary material Table S3), suggesting that Met is not involved in 20E signaling. To confirm the effectiveness of Met RNAi, we performed western blot analysis with rabbit polyclonal antibody against MET. Since

JH repression of AMPs is independent of MET

Fig. 4. 20E potentiates Dpt induction in a dose-dependent manner (A). JH III (B) and the JHa methoprene (C) and pyriproxyfen (D) suppress the 20E-mediated response dose dependently. Results are from luciferase assays with Dpt-luc cells, immune stimulated with PGN; all JH/JHa treatments were performed in combination with 20E (means ± 1 s.e.m.).

Fig. 5. Dpt potentiation by 20E requires at least 18 h of hormone exposure in the presence of PGN (A), but suppression by JHa methoprene is rapid and does not depend on preincubation (B). Results in A are from a northern blot, with quantification of Dpt expression normalized to that of an Rp49 control (northern blot not shown). The x-axis displays the period (in hours) during which cells were exposed to 20E; crude PGN-contaminated lipopolysaccharide (LPS) preparations were used to stimulate the immune response. Results in B are from a luciferase assay with Dpt-luc cells, immune stimulated with PGN; all JH/JHa treatments were performed in combination with 20E. The x-axis displays the different hormone treatments: no hormone, 20E only (for 24 h), or 20E (for 24 h) in the presence of JHa added to cell culture at 4, 6, 8 or 24 h prior to the luciferase assay. Means ± 1 s.e.m.
Hormonal regulation of Drosophila immunity

Fig. 6. 20E blocks S2* cell proliferation, but proliferation is unaffected by JHa methoprene; JHa treatment does not prevent the 20E-mediated block in cell proliferation. Cells were left untreated (filled triangles), or were exposed to 20E (open squares), JHa (filled squares) or both hormones (open triangles); cell counts were monitored every 24 h over the next 3 days. Shown are cell counts (in units of 10^6 cells ml^-1 cell culture media) over time. The result shown is representative of three independent experiments (data not shown).

Fig. 7. (A and B) Northern blotting for Dpt induction shows that ecdysone receptor (EcR) and ultraspiracle (Usp) are both required for the potentiation of Dpt induction by 20E, as determined by RNA interference (RNAi)-mediated silencing using dsRNA. (C) Western blot with mouse monoclonal antibody AB11 against USP; the western blot was performed on the same samples used in the northern blot; RNAi successfully silenced Usp.
endogenous MET levels were low (data not shown), we overexpressed Met with a plasmid expression vector (pMET) and found that RNAi successfully silenced Met (Fig. 9B). Remarkably, when directing RNAi against Met, the JHa methoprene was still able to fully suppress Dpt activity, suggesting that Met does not function in the JH modulation of immunity in Drosophila (Fig. 8C and Fig. 9A; supplementary material Table S3); experiments using JH III yielded similar results (data not shown).

**DISCUSSION**

In insects, 20E and JH coordinate many aspects of growth, development, reproduction, behavior and lifespan (Nijhout, 1994; Riddiford, 1994; Kozlova and Thummel, 2000; Dubrovsky, 2004; Berger and Dubrovsky, 2005; Flatt et al., 2005; Flatt et al., 2006; Tu et al., 2006). Previous evidence indicates that both hormones can individually modulate immunity (Meister and Richards, 1996; Dimarcq et al., 1997; Rolff and Siva-Jothy, 2002; Beckstead et al., 2005). Here, through experiments in Drosophila S2* cells and whole flies, we have shown that 20E and JH exert antagonistic effects on mRNA expression and promoter activity of AMP genes. This is similar to 20E and JH action during midgut remodeling where the JHs methoprene suppresses the expression of genes involved in 20E signaling and 20E-mediated programmed cell death (Parthasarathy and Palli, 2007; Parthasarathy et al., 2008a).

We found that 20E potentiates expression of several AMPs, as previously observed (Meister and Richards, 1996; Dimarcq et al., 1994; Dimarcq et al., 1997; Silverman et al., 2000). We extend previous findings by showing that 20E is a specific hormonal potentiator of AMP induction: upon immune stimulation, 20E enables, in a dose- and time-dependent manner, Dpt induction following immune stimulation. Interestingly, immune potentiation by 20E required at least 18 h of hormone exposure. 20E is known to transcriptionally regulate target genes through enhancers that contain EcR response elements (EcREs). Since this level of transcriptional activation occurs rapidly and since many EcR targets are transcription factors (Thummel, 2002; Yin and Thummel, 2005), it seems likely that 20E mediates the increase in immune responsiveness through secondary or tertiary targets of 20E/EcR signaling.

Although JH has previously been implicated in modulating immunity (Manetti et al., 1997; Hiruma and Riddiford, 1998; Rolff and Siva-Jothy, 2002; Rantala et al., 2003), JH effects on AMP expression have not been investigated. We found that potentiation of Dpt activity by 20E was strongly suppressed by compounds with JH activity (juvenoids). Inhibitory effects were obtained by using not only the JHs methoprene and pyriproxyfen but, importantly, also the natural hormone JH III (methyl epoxy farnesoate). In addition, another product of the larval ring gland and adult corpus allatum (tissues producing JH), the JH precursor methyl farnesoate (Jones et al., 2006; Jones and Jones, 2007), also strongly suppresses 20E induction of Dpt activity (A.G. and T.F., unpublished data). While we consistently observed robust JH- or JHa-mediated suppression of AMP induction in S2* cells, quantitative levels of suppression were quite variable among experiments, presumably due to slight variations in the physiological state of the cells or in luciferase assay conditions. Our dose–response experiments with JH III, MF and JHa suggest that these inhibitory effects are specific hormonal effects; all compounds caused strong suppression of the 20E response at concentrations below 10^{-10} mol l^{-1}. The specificity of these effects is further suggested by our observation that JHa did not block S2* cells from attaining 20E-induced growth arrest and morphological differentiation (see also Wyss, 1976; Cherbas et al., 1989; Echalier, 1997). In contrast to induction by 20E, immune suppression by juvenoids was rapid and did not require preincubation, suggesting that the repression is the result of a primary hormone response.
Moreover, inhibitory effects of juvenoids seen in S2* cells were faithfully mirrored in the fly: in microarrays, GFP reporter assays and northern blot experiments performed on adult flies, JH/JHa acted as powerful immuno-suppressors of AMP expression in vivo. Both JH and 20E act on many target tissues in the fly, including brain, gonads and fat body, the equivalent of the mammalian liver and a major endocrine target tissue (Nijhout, 1994; Riddiford, 1994; Flatt et al., 2005). Since upon systemic infection AMPs are mainly produced in the fat body, it is likely that JH/20E modulation of AMP induction normally takes place in this tissue. Together, our findings suggest that 20E and JH interact antagonistically to regulate immunity in Drosophila.

To understand the mode of JH/20E signaling action in immunity we used RNA interference in S2* cells. We focused on three key genes involved in 20E and JH signaling: ecdysone receptor (EcR), ultraspiracle (Usp) and methoprene-tolerant (Met). 20E signaling requires 20E binding to a heterodimer between EcR and USP (Koelle et al., 1991; Thomas et al., 1993; Yao et al., 1993; Hall and Thummel, 1998). However, 20E signals can also be mediated by EcR homodimers [in vitro (see Lezzi et al., 1999; Lezzi et al., 2002; Grebe et al., 2003)], heterodimers between hormone-receptor 38 (DHR38) and USP (Baker et al., 2003), or non-genomic actions (Wehling, 1997; Elmogy et al., 2004; Srivastava et al., 2005). Confirming the classical model of 20E signal transduction, we found that 20E potentiation of Dpt induction requires both EcR and USP function. When EcR and Usp were silenced with RNAi, JHa still appeared to be able to suppress the residual Dpt induction (see Fig.8A,B); however, this effect was not statistically significant (supplementary material Table S3). Thus, it is possible that the EcR/USP heterodimer is not involved in the JH-mediated immune suppression. On the other hand, we cannot exclude the possibility that EcR/USP integrate both 20E and JH signaling (Fang et al., 2005); under such a model, the EcR/USP heterodimer would be required for both Dpt activation by 20E and its suppression by JH/JHa.

Indeed, the USP part of EcR/USP might be an important mediator of JH signaling since JH can act as a USP ligand and suppress or potentiate 20E-dependent EcR signaling responses (Jones and Sharp, 1997; Jones et al., 2002; Xu et al., 2002; Henrich et al., 2003; Maki et al., 2004; Wozniak et al., 2004; Fang et al., 2005; Jones et al., 2006). For example, JH and 20E can synergistically activate a JH esterase reporter gene (Fang et al., 2005). While these two hormones can activate transcription independently, activation is greater than additive if both hormones are present. In the absence of 20E, activation by JH is through the USP homodimer, whereas activation by 20E in the absence of JH is mediated by EcR/USP (Fang et al., 2005). Notably, when both hormones are present, EcR/USP mediates integration of
JH and 20E signaling, with JH signaling being mediated by the USP part of the 20E-liganded heterodimer (Fang et al., 2005). Thus, our results suggest that EcR/USP is required for 20E signaling in Drosophila immunity, but we cannot rule out the interesting possibility that JH exerts its inhibitory effects by signaling through the USP part of EcR/USP. Future work will be needed to test the requirement of EcR/USP for fat body-specific induction of AMPs in vivo, using dominant negative or RNAi constructs.

Another candidate for the elusive JH receptor is encoded by Met, a basic helix-loop-helix (bHLH)-Per-Arnt-Sim (PAS) transcription factor (Wilson and Fabian, 1986; Shemshedini and Wilson, 1990; Shemshedini et al., 1990; Wilson and Ashok, 1998; Pursley et al., 2000; Wilson et al., 2003). While MET is not a nuclear hormone receptor like EcR or USP, MET binds JH with higher affinity than USP and functions as a JH-dependent transcription factor (Miura et al., 2005). Moreover, Met genetically interacts with the 20E-regulated transcription factor Broad-Complex (Br-C) (Wilson et al., 2006), an important mediator of 20E signaling downstream of EcR, and MET protein interacts with both EcR and USP in GST pull-down assays (Li et al., 2007). However, while Met controls entry into metamorphosis in the beetle Tribolium castaneum, as one would expect if Met encodes a JH receptor (Konopova and Jindra, 2007), Drosophila Met null mutants show normal development (Wilson and Fabian, 1986; Wilson and Ashok, 1998; Flatt and Kawecki, 2004).

To further examine the role of MET in JH signal transduction we directed RNAi against Met in Dpt-luc S2* cells and found that silencing Met does not impair 20E induction of Dpt. Remarkably, we also found that RNAi against Met does not abolish the immunosuppressive action of JH/JHa, despite the involvement of MET in certain JH responses (Miura et al., 2005; Konopova and Jindra, 2007). Similarly, despite its involvement in JH signaling, MET does not seem to be required for JH suppression of 20E action in Tribolium (Parthasarathy and PalIi, 2008; Parthasarathy et al., 2008b). We conclude that MET does not function in the JH regulation of immunity in Drosophila. Thus, it appears that JH suppression of 20E action may be mediated by USP (as part of the ECR/USP heterodimer or as a monomer/homodimer) or by another, as yet unidentified mechanism. While the identity of the JH receptor remains unresolved, the endocrine regulation of Drosophila immunity might provide a powerful model system for studying regulatory cross-talk between JH/20E and for dissecting the elusive JH signaling pathway. Moreover, given the common endocrine-based trade-off between reproduction and immunity in mammals, birds and invertebrates (Muehlenbein and Briebiescas, 2005; Harshman and Zera, 2007; Lawnczak et al., 2007; Miyata et al., 2008), it will be of major interest to study how the reproductive insect hormones JH and 20E interact to co-regulate reproduction and immune function (Flatt et al., 2005).

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Wilson, T. G. (2004). The molecular site of action of juvenile hormone and juvenile hormone insecticides during metamorphosis: how these compounds kill insects. J. Insect Physiol. 50, 111-121.


Wilson, T. G. (2004). The molecular site of action of juvenile hormone and juvenile hormone insecticides during metamorphosis: how these compounds kill insects. J. Insect Physiol. 50, 111-121.


Table S1. Primer sequences (5’ to 3’), with a 5’ T7 promoter sequence (TAATACGACTCACTATAGG) used to generate dsRNA for RNAi silencing in S2* cell culture.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense</th>
<th>Antisense</th>
</tr>
</thead>
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<tr>
<td>EcR</td>
<td>TAATACGACTCACTATAGGGCCAAACTAGTCGAAAGCGATCC</td>
<td>TAATACGACTCACTATAGGGCAGTTGACCTTGCAGCTGAG</td>
</tr>
<tr>
<td>Usp</td>
<td>TAATACGACTCACTATAGGGAAGACGAGCGACAGGAA</td>
<td>TAATACGACTCACTATAGGGAGCAGAATCACCTGGTCGTC</td>
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<tr>
<td>Met</td>
<td>TAATACGACTCACTATAGGGACTGCTGCTGCTTCTCACCCTA</td>
<td>TAATACGACTCACTATAGGGGAGATCTTAACCGCTCGTAA</td>
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<tr>
<td>LacZ</td>
<td>TAATACGACTCACTATAGGGCACTTACCTGGCAACCATCC</td>
<td>TAATACGACTCACTATAGGCCAGAACTGGCGATCGTTCG</td>
</tr>
</tbody>
</table>

EcR, ecdysone receptor; Met, methoprene-tolerant; Usp, ultraspirecle.
Table S2. JH significantly enriches expression of *D. melanogaster* genes responsive to biological stimuli, including immune response genes

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Biological process (FlyBase)</th>
<th>Fold suppression (control/JH)</th>
<th>FlyBase accession</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dorsal-related immunity factor</td>
<td>Defense and immune response, response to fungi, Toll pathway</td>
<td>0.04</td>
<td>FBgn0011274</td>
</tr>
<tr>
<td>Turandot M</td>
<td>Humoral defense mechanism (inferred from sequence similarity)</td>
<td>0.05</td>
<td>FBgn0031701</td>
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<tr>
<td>Tetraspanin 96F</td>
<td>B-cell mediated immunity (inferred from electronic annotation)</td>
<td>0.17</td>
<td>FBgn0027865</td>
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<tr>
<td>Traf3</td>
<td>Defense response (sequence similarity)</td>
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<td>FBgn0030748</td>
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<tr>
<td>CG6662</td>
<td>Defense response (electronic annotation)</td>
<td>0.49</td>
<td>FBgn0035907</td>
</tr>
<tr>
<td>Tetraspanin 74F</td>
<td>Defense response (electronic annotation)</td>
<td>0.50</td>
<td>FBgn0036769</td>
</tr>
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<td>CG6435</td>
<td>Defense response, defense response to bacteria (electronic annotation)</td>
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<td>FBgn0034165</td>
</tr>
<tr>
<td>CG7627</td>
<td>Defense response, response to toxin (electronic annotation)</td>
<td>2.03</td>
<td>FBgn0032026</td>
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<tr>
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<td>Defense response, response to toxin (electronic annotation)</td>
<td>2.04</td>
<td>FBgn0034405</td>
</tr>
<tr>
<td>Immune induced molecule 23</td>
<td>Defense response</td>
<td>2.06</td>
<td>FBgn0034328</td>
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<td>PHGPx</td>
<td>Defense response, response to toxin</td>
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<td>Transferrin 1</td>
<td>Defense response</td>
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<td>FBgn0022355</td>
</tr>
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<td>18 wheeler</td>
<td>Antibacterial humoral response, immune response, Toll-like receptor</td>
<td>3.45</td>
<td>FBgn0004354</td>
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<td>Diptericin B</td>
<td>Antibacterial humoral response (sequence and structural similarity)</td>
<td>3.62</td>
<td>FBgn0034407</td>
</tr>
<tr>
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<td>Melanization, wound healing</td>
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<td>FBgn0029167</td>
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<tr>
<td>Diptericin</td>
<td>Defense response to gram-negative bacteria</td>
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<td>FBgn0004240</td>
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<td>Defense response (electronic annotation)</td>
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<td>CG2736</td>
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<td>takeout</td>
<td>Response to starvation; JH binding protein</td>
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<td>CG18522</td>
<td>Defense response; oxidative stress (electronic annotation)</td>
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<td>FBgn0038347</td>
</tr>
</tbody>
</table>

The majority of these genes (GO:0009607), including several AMPs (marked in bold), are downregulated by juvenile hormone (JH), suggesting that JH is an immuno-suppressor in vivo. Further information on each gene can be found in FlyBase at http://flybase.bio.indiana.edu. Also see Fig. 1 and text for further details.
Table S3. Two-way ANOVA for Dpt promoter activity in Dpt-luc reporter assays shown in Fig. 8

<table>
<thead>
<tr>
<th>Source</th>
<th>F</th>
<th>d.f. num.</th>
<th>d.f. den.</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) EcR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNAi treatment (RNAi vs control RNAi)</td>
<td>17.6</td>
<td>1</td>
<td>20</td>
<td>0.0004</td>
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<tr>
<td>Hormone treatment (20E vs JHa + 20E)</td>
<td>15.8</td>
<td>1</td>
<td>20</td>
<td>0.0008</td>
</tr>
<tr>
<td>RNAi × hormone</td>
<td>58.0</td>
<td>1</td>
<td>20</td>
<td>&lt;0.0001</td>
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<tr>
<td>Contrast, no RNAi, 20E vs JHa + 20E</td>
<td>8.00</td>
<td>1</td>
<td>20</td>
<td>0.06</td>
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<tr>
<td>Contrast, 20E, RNAi vs RNAi control</td>
<td>4.00</td>
<td>1</td>
<td>20</td>
<td>&lt;0.0001</td>
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<tr>
<td>Contrast, 20E + JHa, RNAi vs RNAi control</td>
<td>0.03</td>
<td>1</td>
<td>20</td>
<td>0.87</td>
</tr>
</tbody>
</table>

| (2) Usp |      |           |           |         |
| RNAi treatment (RNAi vs control RNAi) | 14.6 | 1         | 12        | 0.0024  |
| Hormone treatment (20E vs JHa + 20E) | 17.5 | 1         | 12        | 0.0013  |
| RNAi × hormone | 45.8 | 1         | 12        | 0.00002 |
| Contrast, no RNAi, 20E vs JHa + 20E | 0.72 | 1         | 12        | 0.41    |
| Contrast, 20E, RNAi vs RNAi control | 32.0 | 1         | 12        | 0.0001  |
| Contrast, 20E + JHa, RNAi vs RNAi control | 0.07 | 1         | 12        | 0.80    |

| (3) Met |      |           |           |         |
| RNAi treatment (RNAi vs control RNAi) | 0.03 | 1         | 20        | 0.86    |
| Hormone treatment (20E vs JHa + 20E) | 68.7 | 1         | 20        | <0.0001 |
| RNAi × hormone | 0.70 | 1         | 20        | 0.41    |
| Contrast, no RNAi, 20E vs JHa + 20E | 41.7 | 1         | 20        | 0.00003 |
| Contrast, 20E, RNAi vs RNAi control | 27.7 | 1         | 20        | 0.0004  |
| Contrast, 20E + JHa, RNAi vs RNAi control | 0.22 | 1         | 20        | 0.65    |

Dpt, Dipterocercus luciferase; JHa, juvenile hormone analog; RNAi, RNA interference; 20E, 20-hydroxy-ecdysone; d.f. num., degrees of freedom of the numerator; d.f. den., degrees of freedom of the denominator.