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Hormonal regulation of the humoral innate immune response in *Drosophila melanogaster*

Thomas Flatt[1], Andreas Heyland[2], Florentina Rus[3], Ermelinda Porpiglia[3], Chris Sherlock[3,†], Rochele Yamamoto[1], Alina Garbuzov[1], Subba R. Palli[4], Marc Tatar[1] and Neal Silverman[3,*]

[1]Division of Biology and Medicine, Department of Ecology and Evolutionary Biology, Brown University, Providence, RI 02912, USA, [2]Department of Integrative Biology, University of Guelph, Ontario, Canada, N1G 2W1, [3]Department of Medicine, Division of Infectious Disease, University of Massachusetts Medical School, Worcester, MA 01655, USA and [4]Department of Entomology, Agricultural Science Center, College of Agriculture, University of Kentucky, Lexington, KY 40546-0091, USA

*Corresponding author (e-mail: neal.silverman@umassmed.edu)
†Deceased

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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 prophenoloxidase 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 Gram-negative bacteria, activating 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-κB targets, and VDR and its ligand 1,25-dihydroxyvitamin D_3 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 1* (*PPO1*), a gene containing ecdysteroid 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 immunoglobin *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*) (Rolff 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 (JHa). 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,22*R*)-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-(2*E*,4*E*)-11-methoxy-3,7,11-trimethyl-2,4-dodeacdieonate, Sigma (PESTANAL, racemic mixture), 7.9 mmol l^{-1} stock in ethanol]; and the JHa pyriproxyfen {2-[1-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 JHa methoprene and pyriproxyfen are more soluble, more potent and more resistant to *in vivo* degradation than JH III; JHa 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 DD1 [*y*, *w*, *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 *y*, *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 (*Drs*-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 [(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 CO_2 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 CO_2 and their GFP expression visualized under fluorescent (FITC) light with a Zeiss Stemi SV11 dissecting scope; images of individual flies were taken with an AxioCam 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 mm^2; 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 10^6 cells ml^{-1} in six-well tissue culture plates (3 ml of cells per well); after 24 h, cells were split to 10^6 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 10^3 cells μl^{-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 (*Bgl*II–*Eco*RI) 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 10^6 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 10^6 cells ml^{-1} in six-well plates (for northern and western blotting) or plated at 10^3 cells μl^{-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 (10^3 cells μl^{-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

Reagent (Promega; 100 µl per well). Luciferase activity (in relative luciferase units) of samples was assayed with a SpectraMax M5 microplate reader and SoftMax Pro 4.8. software (Molecular Devices, Sunnyvale, CA, USA); samples were automixed for 5 s and luciferase activity determined using the luminescence read mode (top read, three points per well, integration time 1000 ms). For each experiment we used a minimum of three replicate wells per treatment; each experiment was repeated at least four times. Assays combined with RNAi were analyzed with two-way ANOVA implemented in JMP IN 5.1 (Sall et al., 2004), using RNAi (RNAi *vs* control) and hormone (20E *vs* 20E plus JHa) as fixed factors.

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 mol l^{-1} NaCl, 1.5 mmol l^{-1} Na_2HPO_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 mol 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

JH 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 Table S2). 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

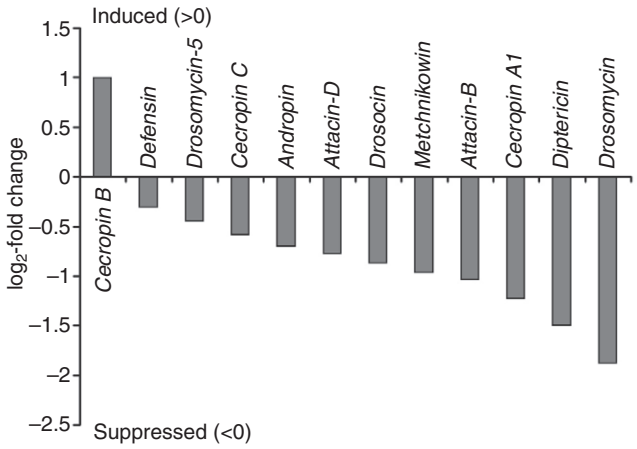

Fig. 1. Juvenile hormone III (JH III) suppresses basal antimicrobial peptide (AMP) expression in whole flies. Shown are log_2-fold change values (JH/control) for 12 AMP transcripts from microarray analyses performed in duplicate. *P<0.05 (Student's t-test). Since the physiological role of JH is not well 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

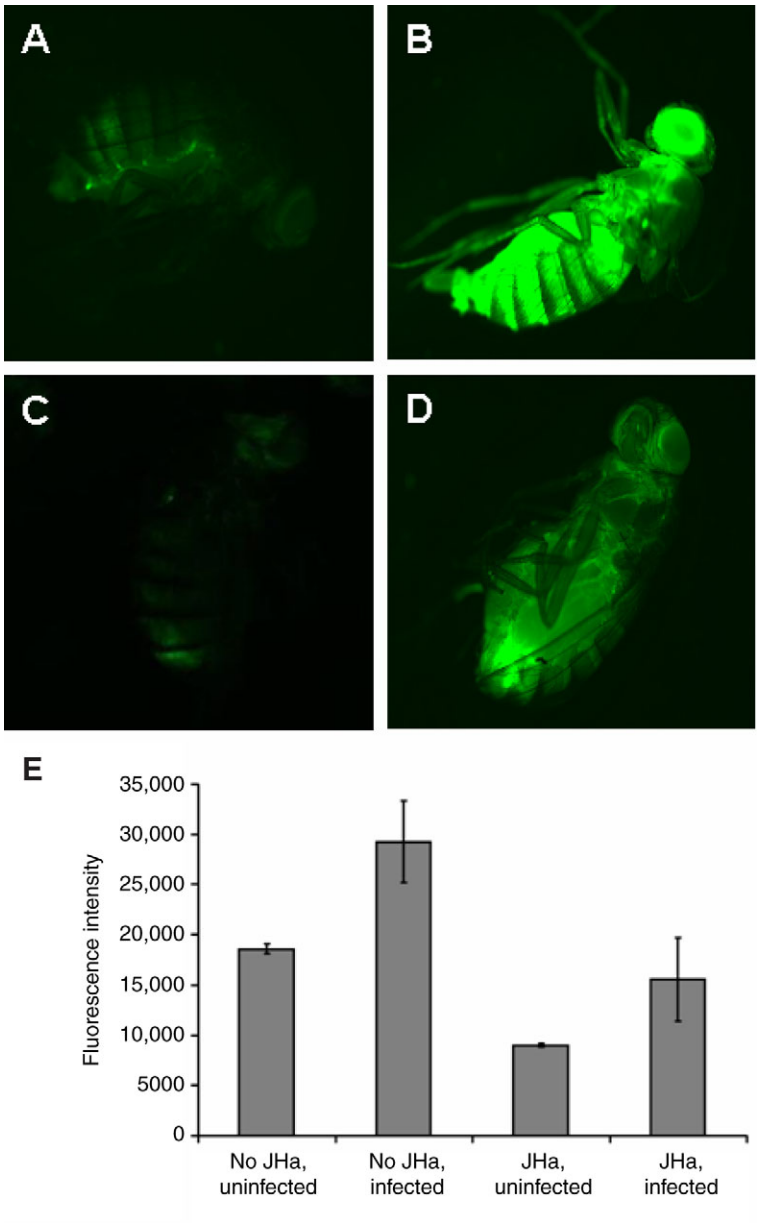

Fig. 2. Juvenile hormone analog (JHa) methoprene reduces expression of *Drosomycin* (*Drs*) in females of the *Drs*-GFP reporter strain DD1. (A) Uninfected (ethanol jabbed), no JHa; (B) infected (*E. coli* jabbed), no JHa; (C) uninfected (ethanol jabbed), JHa; (D) infected (*E. coli* jabbed), JHa; (E) quantification of GFP signals from images (means ±1 s.e.m.; sample size per group, $N=3$). Note the strong autofluorescence in the ovaries (e.g. in D). Qualitatively similar results were obtained with a GFP reporter for *Diptericin* (*Dpt*-GFP; DIG) and in a northern blot on *Dpt* mRNA in *y, w* females (data not shown), suggesting that JH/JHa acts as a suppressor of AMP induction *in vivo*.

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 immuno-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.

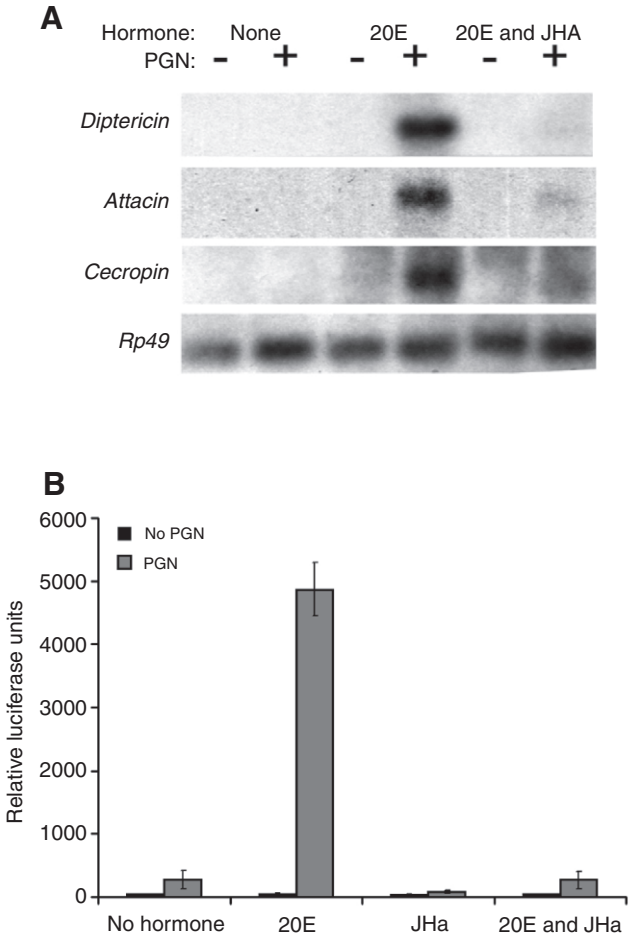

Fig. 3. 20-hydroxy-ecdysone (20E) and JHa methoprene have antagonistic effects on AMP expression. (A) Northern blot monitoring expression of AMPs *Dpt*, *Attacin* and *Cecropin* in S2* cells treated with peptidoglycan (PGN) or untreated, and with different combinations of hormones. *Rp49*, control (encoding ribosomal protein RP49). (B) Luciferase assay in S2* cells stably transfected with a *Dpt*-luciferase reporter construct (means ±1 s.e.m.).

We found that cells treated with both JHa (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.

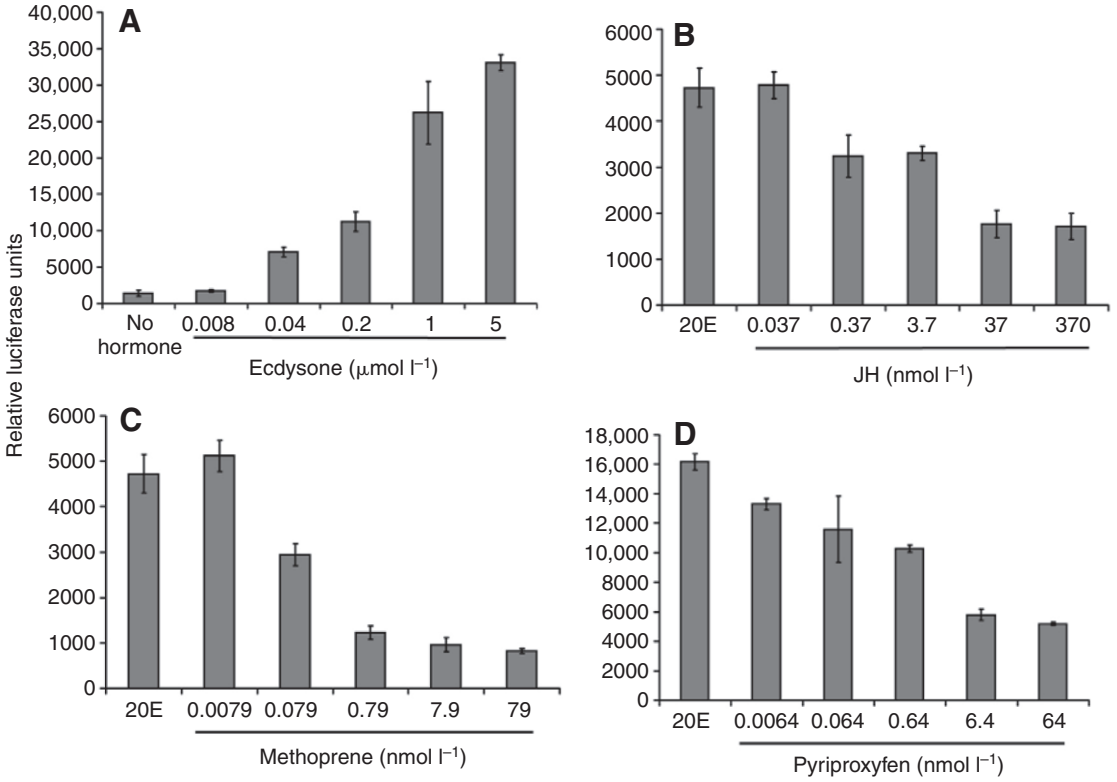

Fig. 4. 20E potentiates *Dpt* induction in a dose-dependent manner (A). JH III (B) and the JHa methoprene (C) and pyriproxyfen (D) suppress the 20E-mediated response dose dependently. Results are from luciferase assays with *Dpt*-luc cells, immune stimulated with PGN; all JH/JHa treatments were performed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

JH repression of AMPs is independent of MET

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

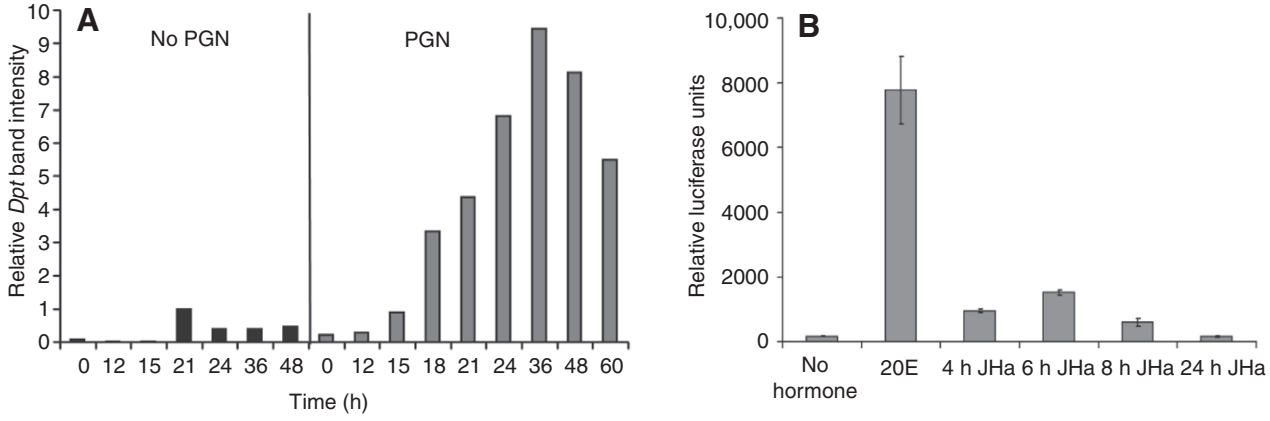

Fig. 5. *Dpt* potentiation by 20E requires at least 18 h of hormone exposure in the presence of PGN (A), but suppression by JHa methoprene is rapid and does not depend on preincubation (B). Results in A are from a northern blot, with quantification of *Dpt* expression normalized to that of an *Rp49* 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.

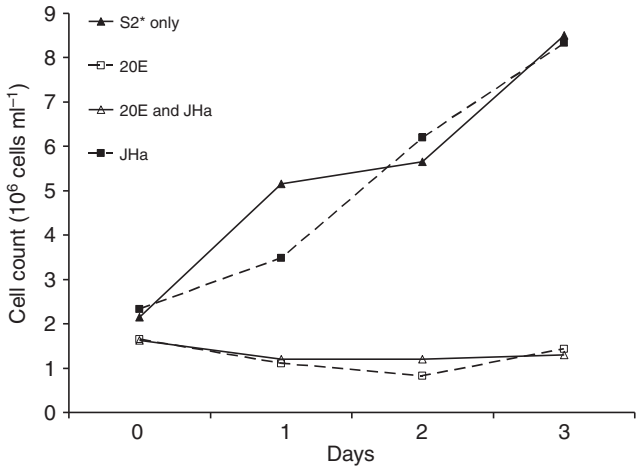

Fig. 6. 20E blocks S2* cell proliferation, but proliferation is unaffected by JHa methoprene; JHa treatment does not prevent the 20E-mediated block in cell proliferation. Cells were left untreated (filled triangles), or were exposed to 20E (open squares), JHa (filled squares) or both hormones (open triangles); 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).

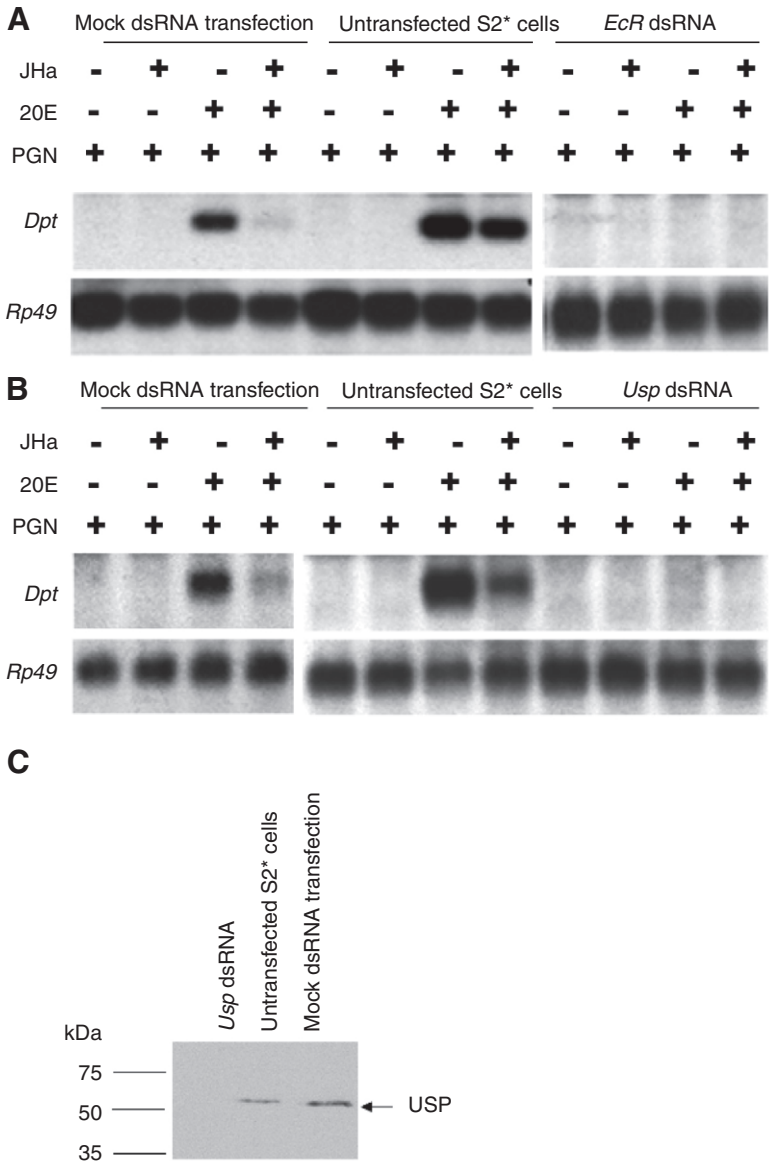

Fig. 7. (A and B) Northern blotting for *Dpt* induction shows that *ecdysone receptor* (*EcR*) and *ultraspiracle* (*Usp*) are both required for the potentiation of *Dpt* induction by 20E, as determined by RNA interference (RNAi)-mediated silencing using dsRNA. (C) Western blot with mouse monoclonal 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).

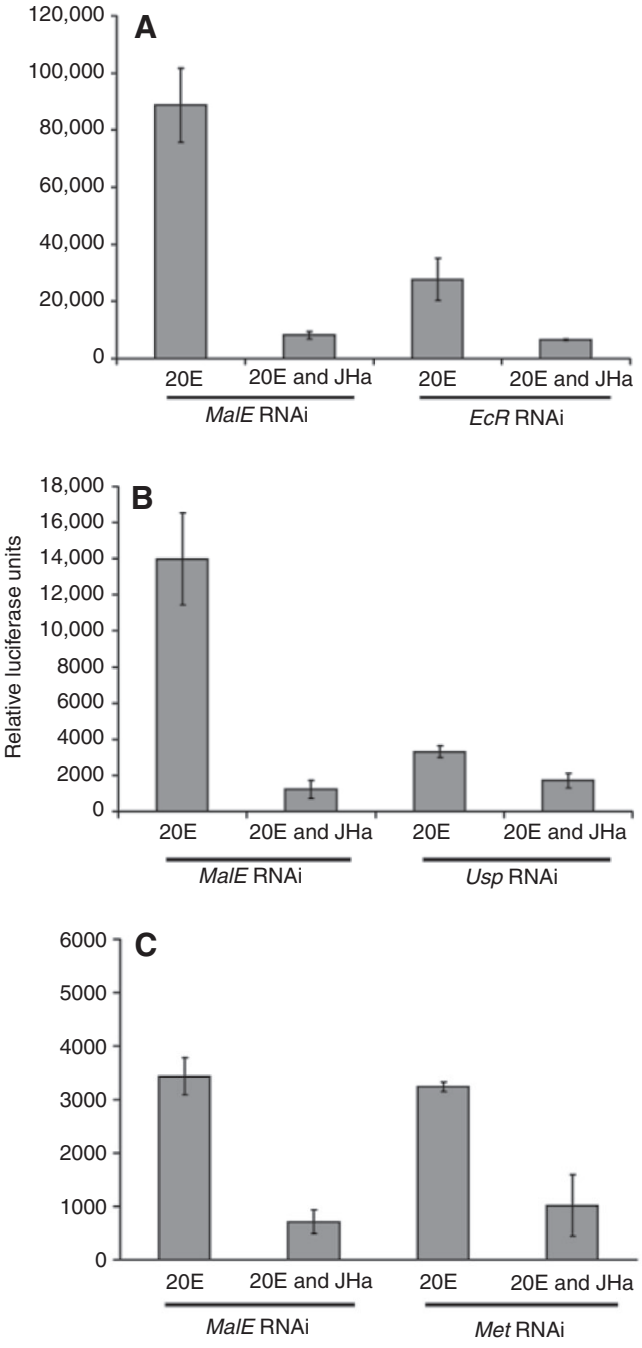

Fig. 8. *Dpt* promoter activity with RNAi-mediated silencing (dsRNA) directed against *EcR* (A), *Usp* (B) and *Met* (C). *E. coli MalE*, control (encoding maltose binding protein). Silencing *EcR* and *Usp* abolishes the 20E response; JHa methoprene seems to suppress the weak *Dpt* induction that occurs in *EcR* or *Usp* knock-down cells; however, this effect is not significant (supplementary material Table S3). In contrast, silencing *Met* does not impair the 20E response, and JHa is fully effective in suppressing immune induction by 20E. 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.).

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 JHa 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 JHa 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.

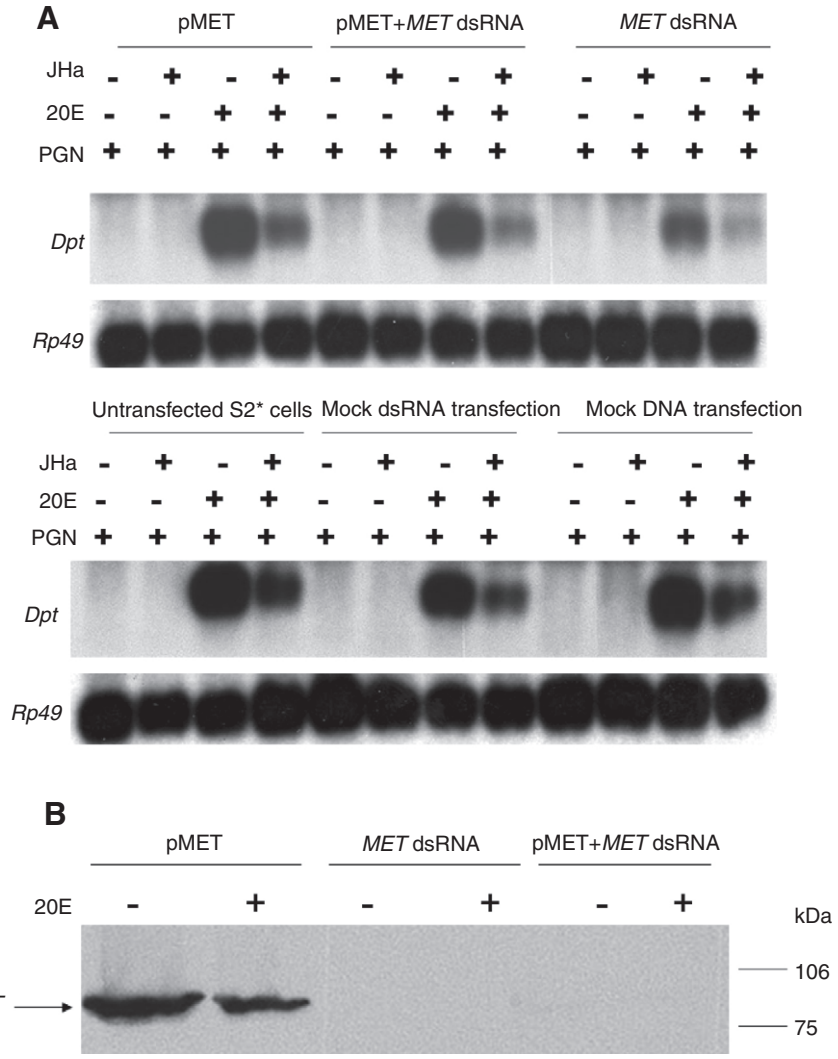

Fig. 9. (A) Northern blotting for *Dpt* shows that *Met* is not required for induction of *Dpt* expression by 20E; notably, JHa methoprene in the presence of 20E is able to fully suppress *Dpt* expression even when *Met* function is silenced; pMET refers to cells transfected with *Met* expression vector plasmid. (B) Western blot with rabbit polyclonal antibody against MET; the western blot was performed on the same samples used in the northern blot; RNAi successfully silenced *Met*.

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 Palli, 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 Bribiescas, 2005; Harshman and Zera, 2007; Lawniczak 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).

LIST OF ABBREVIATIONS

AMPs	antimicrobial peptides
DD1	*Drosomycin*-GFP strain
DIG	*Diptericin*-GFP reporter strain
Dpt	*Diptericin*
Drs	*Drosomycin*
20E	20-hydroxy-ecdysone
EcR	*ecdysone receptor*
FBS	fetal bovine serum
GO	gene ontology
GR	glucocorticoid receptor
IMD	immune deficiency pathway
JH	juvenile hormone
JHa	juvenile hormone analog
LPS	lipopolysaccharide
Met	*methoprene-tolerant*
NF-κB	nuclear factor kappa B
PGN	peptidoglycan
PO	phenoloxidase
RNAi	RNA interference
S2* cells	Scheider S2* cells
Usp	*ultraspiracle*
VDRs	vitamin D receptors

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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

EcR sense	TAATACGACTCACTATAGGGCCAACTAGTCGAAGCGATCC
EcR antisense	TAATACGACTCACTATAGGGCAGTTGACCTTGCAGCTGAG
Usp sense	TAATACGACTCACTATAGGGATAGACAAGCGGCAGAGGAA
Usp antisense	TAATACGACTCACTATAGGGAGCAGAATCACCTGGTCGTC
Met sense	TAATACGACTCACTATAGGGACTGCTGCTTCCTCACCCTA
Met antisense	TAATACGACTCACTATAGGGCGATCTTAACCCGCTCGTAA
LacZ sense	TAATACGACTCACTATAGGGCATTATCCGAACCATCC
LacZ antisense	TAATACGACTCACTATAGGGCAGAACTGGCGATCGTTCG

EcR, *ecdysone receptor*; *Met*, *methoprene-tolerant*; *Usp*, *ultraspiracle*.

Table S2. JH significantly enriches expression of *D. melanogaster* genes responsive to biological stimuli, including immune response genes

Gene name	Biological process (FlyBase)	Fold suppression (control/JH)	FlyBase accession
Dorsal-related immunity factor	Defense and immune response, response to fungi, Toll pathway	0.04	FBgn0011274
Turandot M	Humoral defense mechanism (inferred from sequence similarity)	0.05	FBgn0031701
Tetraspanin 96F	B-cell mediated immunity (inferred from electronic annotation)	0.17	FBgn0027865
Traf3	Defense response (sequence similarity)	0.43	FBgn0030748
CG6662	Defense response (electronic annotation)	0.49	FBgn0035907
Tetraspanin 74F	Defense response (electronic annotation)	0.50	FBgn0036769
CG6435	Defense response, defense response to bacteria (electronic annotation)	2.02	FBgn0034165
CG7627	Defense response, response to toxin (electronic annotation)	2.03	FBgn0032026
JH expoxide hydrolase 2	Defense response, response to toxin (electronic annotation)	2.04	FBgn0034405
Drosocin	Defense response to gram-positive and -negative bacteria	2.05	FBgn0010388
Immune induced molecule 23	Defense response	2.06	FBgn0034328
PHGPx	Defense response, response to toxin	2.13	FBgn0035438
Ejaculatory bulb protein III	Response to virus	2.17	FBgn0011695
CG12780	Gram-negative bacterial binding, defense response	2.34	FBgn0033301
Metchnikowin	Antibacterial and antifungal humoral response	2.35	FBgn0014865
CG1702	Defense response, response to toxin (electronic annotation)	2.49	FBgn0031117
CG6426	Defense response to bacteria (electronic annotation)	2.58	FBgn0034162
CG5397	Defense response (electronic annotation)	2.61	FBgn0031327
CG13422	Defense response to gram-negative bacteria (electronic annotation)	2.64	FBgn0034511
CG10307	Defense response (electronic annotation)	2.66	FBgn0034655
Transferrin 1	Defense response	2.99	FBgn0022355
18 wheeler	Antibacterial humoral response, immune response, Toll-like receptor	3.45	FBgn0004364
Diptericin B	Antibacterial humoral response (sequence and structural similarity)	3.62	FBgn0034407
Hemolectin	Melanization, wound healing	3.63	FBgn0029167
Diptericin	Defense response to gram-negative bacteria	3.67	FBgn0004240
CG1681	Defense response (electronic annotation)	3.76	FBgn0030484
CG2736	Defense response	3.78	FBgn0035090
CG8336	Defense response	3.79	FBgn0036020
takeout	Response to starvation; JH binding protein	4.18	FBgn0039298
CG18522	Defense response; oxidative stress (electronic annotation)	4.80	FBgn0038347

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

Source	*F*	$d.f._{num.}$	$d.f._{den.}$	*P*
(1) *EcR*				
RNAi treatment (RNAi *vs* control RNAi)	17.6	1	20	0.0004
Hormone treatment (20E *vs* JHa + 20E)	46.3	1	20	<0.0001
RNAi × hormone	15.8	1	20	0.0008
Contrast, no RNAi, 20E *vs* JHa + 20E	58.0	1	20	<0.000001
Contrast, RNAi, 20E *vs* JHa + 20E	4.00	1	20	0.06
Contrast, 20E, RNAi *vs* RNAi control	33.4	1	20	<0.0001
Contrast, 20E + JHa, RNAi *vs* RNAi control	0.03	1	20	0.87
(2) *Usp*				
RNAi treatment (RNAi *vs* control RNAi)	14.6	1	12	0.0024
Hormone treatment (20E *vs* JHa + 20E)	29.0	1	12	0.0002
RNAi × hormone	17.5	1	12	0.0013
Contrast, no RNAi, 20E *vs* JHa + 20E	45.8	1	12	0.00002
Contrast, 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	0.000003
Contrast, RNAi, 20E *vs* JHa + 20E	27.7	1	20	0.00004
Contrast, 20E, RNAi *vs* RNAi control	0.22	1	20	0.65
Contrast, 20E + JHa, RNAi *vs* RNAi control	0.52	1	20	0.48

Dpt, *Diptericin*; luc, 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.