THE EMBO JOURNAL

Manuscript EMBO-2012-81423

Ecdysone Triggered PGRP-LC Expression Controls *Drosophila* Innate Immunity

Florentina Rus, Thomas Flatt, Kamna Aggarwal, Mei Tong, Kendi Okuda, Anni Kleino, Elisabeth Yates, Marc Tatar, Neal S. Silverman

Corresponding author: Neal S. Silverman, University of Massachusetts

Review timeline:

Submission date:	16 March 2012
Editorial Decision:	30 April 2012
Revision received:	20 December 2012
Editorial Decision:	23 January 2013
Revision received:	02 April 2013
Accepted:	04 April 2013

Editor: Karin Dumstrei

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 30 April 2012

Thank you for submitting your manuscript to the EMBO Journal. I am sorry for the delay in getting back to you with a decision, but I have now received the full set of referee comments.

As you can see, there is an interest in this paper, but from the comments provided it is also clear that much further work would be needed in order to consider publication here. There are several issues raised, which are clearly outlined below. What is definitively needed is more in vivo data to support the role of Ecdysone signaling in the immune response. This means additional mutational analysis and to look at the ability of flies to clear microbes rather than to measure survival of flies (referee #1 - please also take into consideration the tolerance vs resistance issues that this referee is raising). Also better quantification is needed and you need to extend the findings to several microbes. Should you be able to address the concerns raised in full, with the inclusion of additional data, then we would consider a revised version. I realize that addressing the concerns in full requires a significant amount of additional work, but that is the level of insight needed for publication here. I can extend the revision time to 6 month is that is helpful. I should also add that should you not be able to extend the analysis along the lines indicated by the referees then it is in your best interest to seek publication elsewhere at this stage.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

———

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

I generally accept the authors' conclusions that 20-hydroxy ecdysone can act as an immune modulator in cell culture and actual living flies. The mechanistic results here seem clear even if they are complicated. I would like to see the authors replace all relative arguments that are voiced with adjectives denoting the size of an effect replaced with quantitative measurements because what is a "big" effect to one scientist might be a "modest" effect to another. The authors run into trouble when they try to discuss the biological relevance by studying this effect in vivo. I'm not sure what to do about this as the mechanistic story makes sense but the in vivo work will require substantially more work to meet my standards.

At issue is how one assays the importance of an immune response in a fly. If the authors use the transcription of antimicrobial peptide genes as an assay, I would expect them to assay the activity of these genes in the fly. The only way to do this, that I know, is to measure the ability of the fly to clear microbes. The authors don't report this in the main figures. Instead they measure the survival of the flies and this is an inappropriate assay.

The authors did not reference the literature that tries to relate microbe load to the health of the animal. Why do flies die from infections? We don't know the answer in most cases. In one case I know, the flies waste from the infection and changes in the fly that control the rate of wasting affect the mean time to death of the fly but don't change the microbe load; this demonstrates that not all changes that affect survival affect microbe clearance. Ecological immunologists characterize the dose response curve of health to microbe load as "tolerance". These effects don't seem to be rare - when people look in genetic screens, they are picked up about as frequently as changes in the ability to control bacterial growth.

Given that ecdysone is a hormone that regulates many physiologies in the fly including egg production, AMP expression and metamorphosis, doesn't it seem plausible that it is also regulating energy stores? Even if energy stores aren't being regulated, it seems plausible that ecdysone is altering the tolerance curve instead of or in addition to the resistance of the host.

The story is more complicated than this; once you start measuring both resistance and tolerance to infections you find that single fly mutants react differently to different pathogens. For example, you might find a mutation that makes a fly less resistant to one microbe but more tolerant to another. A way of explaining this would be that in one case the immune response is required to kill microbe A, however the immune response does little against microbe B and if this immune response is engaged it will increase pathology. The risk in testing only one microbe is that you can railroad your results into seeming like you are getting just one phenotype out of a manipulation. As many as 5 different immune phenotypes have been reported for a single fly mutation when one challenges the fly with enough microbes. Therefore, I'm wary of manuscripts that simply test one microbe because you have to worry that other microbes would give a different result. If the authors were primarily interested in studying Erwinia, it would be OK to test just Erwinia but if they are interested in generally studying the fly's immune response, it seems advisable to test several microbes.

Throughout the manuscript the authors use adjectives reporting relative measurements:
"... that were modestly but significantly increased upon hormone treatment"
"PGRP-LC expression is roughly coincident with the developmental pulses of 20E"
"In fact, ectopic expression of PGRP-LCx in these cells led to modest
expression of these AMP genes in the absence of any immune stimulus."
"which robustly affects only a subset of AMP genes"
"Depletion of Eip75B, by contrast, modestly increased PGRP-LC levels, consistent with the higher levels of Dpt expression observed with knockdown of this gene"
Who are we to say that an effect is "strong" or "minor," "modest" or "indecent?" The authors

certainly aren't the only people who write like this and I've been irritated by this behavior for a while. My impression is that authors generally write about their own effects as being "strong" and those of competitors as being "weak" even when they are exactly the same size. The safest way around this problem is to simply list the relative numerical sizes of the responses and their statistical significance and let evolution be the judge of whether something is weak or strong. For example, a 1% increase in fitness might seem "small" in the lab but could rapidly lead to a selective sweep in the wild.

Perhaps this comment runs counter to my argument above, but the authors could make figure 1A more useful if they log transformed the X axis. This way a change of 195x in gene expression would take up more than 5% of the left hand side of the figure and one could more readily interpret the graph without using an inset. Why is there a -1000 plotted on the X axis of 1A when nothing is plotted below this zero?

For figure 1B, could the authors please apply ANOVA and report which transcript levels fall into different groups. This applies to all quantitation graphs.

I am also concerned about some of the ecdysone genes the authors are surveying and the assumption that their phenotype depends upon their effects upon antimicrobial peptide production. I'm worried about pannier and serpent in particular as both have previously been implicating in regulating another arm of the fly's immune response, namely the cellular immune response. There is a Lemaitre paper (PNAS March 28, 2000 vol. 97 no. 7 3376-3381) which looked at Erwinia induction of immune responses in flies with altered hemocytes that argued the hemocytes were required for diptericin expression. This doesn't mean that old paper is true but the result is out there. The paper raises an alternative explanation of figure 4A - that alteration of hemocyte activity reduces diptericin transcript levels.

Referee #2 (Remarks to the Author):

This manuscript reports several potentially interesting findings related to a humoral control of innate immunity:
i. ecdysone (20E) controls the expression of PGRP-LC in S2* cells and the ectopic expression of PGRP-LC is sufficient to bypass a 20E requirement for the expression of some antimicrobial peptide (AMP) genes, e.g., Attacin A, Cecropin A, Defensin;

ii. 20E exerts a second level of control in S2* for the expression of some AMP genes, e.g., Diptericin. This control presumably occurs downstream of Relish activation;

iii. many 20E target genes, which encode transcription factors, appear to be required for 20E-mediated expression of PGRP-LC in S2* cells. However, only serpent and br-c appear to be required for the second level of control;

iv. 20E-dependent signaling appears to be required for AMP signaling in vivo and possibly resistance against bacterial infections.
While the cell culture work appears to be solid, the in vivo part is somewhat disappointing in that it does not address the complexity of signaling and does not establish whether hormonal control is mediated through PGRP-LC expression as in S2* cells. Also, it would be important to determine whether the second level of control is relevant in vivo. Finally, depending on the AMP gene under consideration, the kinetics are totally different and here the expression of only one gene at one time point is reported.
Thus, this reviewer suggests repeating in vivo some of the experiments done in cell culture, namely whether PGRP-LC ectopic expression may rescue the defective immune response observed in some 20E target gene mutants. This reviewer is aware that these experiments are difficult in that a mild overexpression of PGRP-LC is sufficient to trigger signaling independently of an immune challenge. Thus, adequate controls will be essential. As regards the so-called "realized" immune response, it would be important to check that the bacterial titer is indeed increasing, that is that flies are indeed succumbing to bacteremia. Also, the use of Ecc15 alone may blur the sensitivity of the assay as it may be too pathogenic. Indeed, E. coli would appear to be a much better choice. Even though some

conflicting results have been published in the literature, it appears that this bacterium is unable to kill PGRP-LC mutants. Thus, if the 20E effect is solely mediated via PGRP-LC expression, one may expect that mutants for some of the 20-E target genes may be only weakly susceptible to this challenge. It would then become very meaningful to compare br-c and srp mutants against one of the Eip mutants.
The Lemaitre 1996 Cell paper and some subsequent reports established that there were several categories of AMP genes. Interestingly, those with a short-term kinetics after an immune challenge (Attacin-A, Cecropin A and Defensin) are also those that appear to depend both on the IMD and Toll pathways when challenged with a mixture of E. coli and M. luteus. How is the kinetics of these AMP genes affected when 20E signaling is disrupted?

Minor points
1. It is interesting that the inactivation of many 20E target genes leads to a phenotype. Have the authors determined whether the expression of other 20E-dependent genes is affected when a single gene is targeted?

2. The authors mention the existence of delay between hormonal signaling and PGRP-LC expression. Does this also apply to embryogenesis (Fig. S1)?

3. p9: when referring to dominant acting mutants, this reviewer supposes the authors mean dominant-negative?

4. As regards the experiments with 20E target genes RNAi in S2* cells, data are only provided for AMPs that are under dual control. What happens to others?

5. If this reviewer recalls correctly, there are studies in which for instance Attacin was induced by an immune challenge in cultured cells without any 20E treatment. What is the difference due to? Different cell lines? Alternatively, have the authors attempted to stimulate their cells with heat-killed bacteria rather than purified PGN?

6. p10, last sentence: the authors refer to CecA1 data that, as noted above, are not provided in this manuscript.

7. There are many plots in which statistical significance is not indicated; this appears especially important for Fig. S9 in which some error bars appear to be rather large. Which statistical procedure was used?

8. The results shown in Fig. 3 and Fig. S5 are rather different as regards the levels of induction induced by PGN in the absence of 20E. What is this due to? Different experiments or difference due to the use of two different techniques? Have the authors checked the data yielded by qRT-PCR and nanostring analysis on the same samples?

9. p5: are the references provided for the sentence on the role of PGRP-LC in innate immunity really the most relevant ones as only one study reported some in vivo data?

Referee #3 (Remarks to the Author):

The interest of this paper is based on two important results, firstly that ecdysone regulates transcription of PGRP-LC, the receptor upstream of the IMD pathway, and secondly that AMP (antimicrobial peptide gene) expression downstream of PGRP-LC is itself is under ecdysone regulation, with distinctive groups of AMPs responding more or less exclusively to ecdysone-dependent transcription factors. Taken together, the results presented in this paper clearly demonstrate the strong coupling between ecdysone-mediated transcriptional control and the IMD pathway.
Overall, we deem this study worthy of publishing, based on its solid findings on hormonal control of

the immune response which extend our knowledge of this poorly characterized aspect of the immune response. However, a weakness of this study is its lack of mechanistic exploration on how ecdysone signals integrate at the level of either the PGRP-LC or different classes of AMP promoters. We feel that additional results towards these points would strengthen the paper's message and raise its suitability for a journal like EMBO.

Therefore, we list the following major recommendations:

1) Determine the mechanistic basis of ecdysone regulation on PGRP-LC and at least one of each group of AMP genes by an in-depth in silico and/or in vivo promoter analysis.
2) As the experiment shown in Figure 6 is crucial, we would recommend to reproduce it with an additional, fat body-specific driver such as c546-Gal4 (combined to a thermo-sensitive repressor Gal80 to avoid developmental effects) and verify that the drivers themselves are ecdysone-independent. Analysing Drs expression (or any other Toll-dependent AMP) after infection with Lys-type bacteria would be an interesting control. I would also recommend to include a 3-6-12h time points with a subset of RNAi lines to test the effect of ecdysone at different time points..
3) It would be interesting to test whether ecdysone affects the IMD pathway in all tissues or whether its action is restricted to the fat body. Analysing the local immune response in the gut of adults would answer this question.

Minor suggestions

ï Please add statistical analysis to Fig. 4 (text mentions "mild effects" - are they non-significant?), Fig. 5, Fig. 6 (text mentions "markedly reduced" expression -significant?), Fig. S8 (text mentions "no effect" - is reduction observed with Hsf and luna non-significant?), Fig. S9 (text mentions "significantly reduced Dpt expression" - P-value?)
ï The normalization procedure used in Fig. 3 is unclear - are S2* cells and PGRP-LCx-FLAG cells normalized separately to their respective Dpt levels induced with 20E and PGN?
ï Since the counteracting effect of JH on 20E is mentioned in the discussion, it would add value to Fig. S1 to see also the JH peaks illustrated as bars above the graph.
ï Figures S2 and S3 include stimulation with PGN for 10 min, but this is not discussed anywhere in the text. Why is this included? There is no explanation as to why 10 min exposure to PGN seems to increase PGRP-LCx expression (is PGN added to cells pulling down PGRP-LC along with the IP?)
ï For readers not familiar with the nuclear receptor literature, it would be informative to add more details about the selection criteria used to choose the twelve 20E-inducible transcription factors (section 4 of Results). Likewise, the discussion mentions 64 ecydsone-inducible TFs but only 11 were analysed. Is anything known (from this study) about the 53 remaining? Are they silent with regards to IMD signalling?
ï The observations reported in this paper are likely to explain a number of described mutations that seem to indirectly affect the IMD pathway, or the fluctuations in AMP gene expression observed in various microarrays. Optionally, this could be included in the discussion.
ï In case the microarray data are deposited in GEO or accessible elsewhere, please provide a link.

Very minor corrections
Results:

1st section:
...we and others have demonstrated that the 20E modulates...
2nd section:
Occurring twice: PGRP not PRGP
...expression at its natural loci locus.
3rd section:
...the immune-induced expression of a distinct subset of AMP genes...
4th section:
"As expected, AMP genes were not induced in the parental S2* cells" - shouldn't it be "reduced"?
5th section:
EcRNP5219, which carries a P-insertion in an EcR intron,...
At the fully restrictive temperature (29{degree sign}C), the DTS-3 mutant...
...DTS-3/+ mutant females flies... (occurs twice)
By expressing transgenic hairpin-RNAs with the Gal4/UAS system...

Please use "Erwinia carotovora carotovora 15" or Ecc15, not Erwinia carotovora

Discussion:
"ROR " not RORa
To avoid confusion, please use the same abbreviation for "Rev-Erb " and "Eip75B" throughout (pick one - unfortunately the literature is not consistent on either gene)
...,promoting production of IL-17, Il-21 ...
...shown to trigger a systemic IMD response following a local gut infection...
"GR" define - has not come up before
...regulatory network delineated in this study is part of the neuroendocrine-immuno immuno-neuroendocrine axis...
Materials and Methods

Co-IP
Missing/erroneous symbol in "Image Reader LAS-4000"
RNAi
...stimulated with PGN for an additional 6 hours...
Confocal microscopy
...YFP-Relish and single stables cells expressing the YFP-Relish...
...35-mm glass-bottomed culture plates.
Fly strains and survival experiments
The first paper establishing the Ecc15 model of infection is: Basset A, Khush RS, Braun A, Gardan L, Boccard F, Hoffmann JA, Lemaitre B. The phytopathogenic bacteria Erwinia carotovora infects Drosophila and activates an immune response. Proc Natl Acad Sci U S A. 2000 Mar 28;97(7):3376-81.

General:
Please verify all occurrences of {degree sign}C and use the conventional {degree sign} symbol, not o in superscript
(1 occurrence in Mat&Met, 1 in Suppl Fig legends, graphs in Fig. S9)

Figure Legends

Fig.4: Eip93F missing from title - please add if omitted involuntarily
Fig.6: PGRP-LC missing from list of RNAi (as above)

Figures
Fig. 1 A: effete not effette

Suppl. Figure legends
Fig. S1: The legend says "time shown at bottom" but is displayed on top in Fig. S1.
Fig. S2: Samples were untreated or treated with 20E...
Fig. S10: EcR, Eip75B, PGRP-LC missing from title, and PGRP-LC missing from legend, please add if omitted involuntarily
...showing survival of the same males genotypes after infection...

Suppl. Figures
Fig. S3: please align - and + as in Fig. S2
Fig. S4 D: PGRP-LCx-FLAG-YFP-Relish cells

1st Revision - authors' response 20 December 2012

Point-by-Point Response to Reviewer Comments

Referee #1:

I generally accept the authors' conclusions that 20-hydroxy ecdysone can act as an immune modulator in cell culture and actual living flies. The mechanistic results here seem clear even if they are complicated. I would like to see the authors replace all relative arguments that are voiced with adjectives denoting the size of an effect replaced with quantitative measurements because what is a "big" effect to one scientist might be a "modest" effect to another.

In response to this suggestion, we replaced the adjectives denoting the size of an effect and added P-values to support the significance of the effects reported.

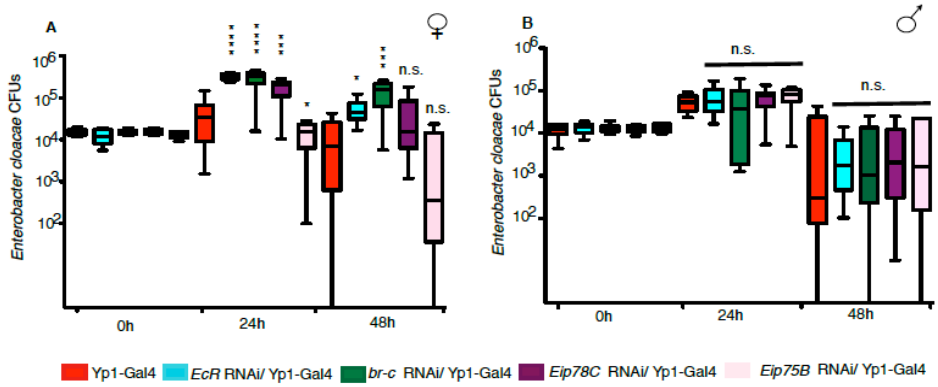
At issue is how one assays the importance of an immune response in a fly. If the authors use the transcription of antimicrobial peptide genes as an assay, I would expect them to assay the activity of these genes in the fly. The only way to do this, that I know, is to measure the ability of the fly to clear microbes.

Indeed, several studies (citations now included) have identified genes whose mutation leads to a decreased ability of the flies to survive infection but not their ability to clear bacteria, suggesting that these genes affect tolerance defense mechanisms rather then the ability to kill and clear microbial infection, referred to as resistance mechanisms. However, the IMD pathway, and the production of AMPs, is most closely linked to resistance mechanisms, as they directly kill microbes. Consistent with this idea, we now show that interfering with the ecdysone signaling pathway in the fat bod affects clearance of *Erwinia carotovora carotovora 15* (Figure S10), in addition to reduced AMP induction and survival. On the other hand, RNAi targeting *Eip75B,* which causes elevated expression of *PGRP-LC* and AMPs (Figure 6) as well as increased survival following *Ecc15* infection, showed an increased ability to clear this infection (Figure S10). These additional data demonstrate that hormone signaling, likely through its effects on the IMD pathway, affects the ability of animals to resist infection and kill microbes.

The story is more complicated than this; once you start measuring both resistance and tolerance to infections you find that single fly mutants react differently to different pathogens. The risk in testing only one microbe is that you can railroad your results into seeming like you are getting just one phenotype out of a manipulation if they are interested in generally studying the fly's immune response, it seems advisable to test several microbes.

We agree with the reviewer, it is dangerous to make generalization based on only one condition. For that reason, we have analyzed not just the EcR but 12 EcR-induced transcription factors in our RNAi studies, both in cell culture and adult animals. In addition, the manuscript presents data with 2 different microbes (as well as bacterial peptidoglycan in the cell-based studies). We show that AMP induction (via the IMD pathway) depends on 20E signaling

following PGN stimulation of S2* cells or *E. coli* infection of adult flies. We also show that survival and (now) clearance of another gram-negative bacteria, *Ecc15,* are similarly dependent on 20E signaling in adult flies. It is established that the IMD is critical for the defense against *Ecc15* (Aggarwal et al., 2008; Kim et al., 2006). In addition, we provide below data showing that the clearance of a 3rd gram-negative bacteria, *Enterobacter cloacae,* is similarly dependent on 20E signaling. However, this data is not currently included in the manuscript because we feel it adds little to our conclusion that IMD-mediated immune defense is critically regulated by 20E signaling. We do not claim that other immune pathways, or other pathogens not controlled by the classic IMD pathway, are also regulated through steroid hormones.



Effect of transcription factors RNAi on growth of *Enterobacter cloacae*

Growth of *Enterobacter cloacae* in adult flies depleted of *EcR*, *br-c*, *Eip78C* or *Eip75B* at 0h, 24h, 48h following infection. 10 individual female (A) or male (B) flies were homogenized and serially diluted and plated on LB-nalidixic acid plates. CFUs were counted after overnight culture at 37C. *P<0.05, **P<0.005, ***P<0.0005, ****P<0.0001, were calculated by unpaired t-test by comparison to *Ecc15* infected control (*Yp1-GAL4*) flies.

Perhaps this comment runs counter to my argument above, but the authors could make figure 1A more useful if they log transformed the X axis. This way a change of 195x in gene expression would take up more than 5% of the left hand side of the figure and one could more readily interpret the graph without using an inset. Why is there a -1000 plotted on the X axis of 1A when nothing is plotted below this zero?

Due to methods used to calculate expression levels in the Affymetrix microarray platform, expression level of PGRP-LC in the absence of hormonal treatment appears as negative value, therefore a -1000 appeared on X-axis. However, we have now manually changed this value to zero (as expression cannot be negative) and the X-axis no longer includes these negative values.

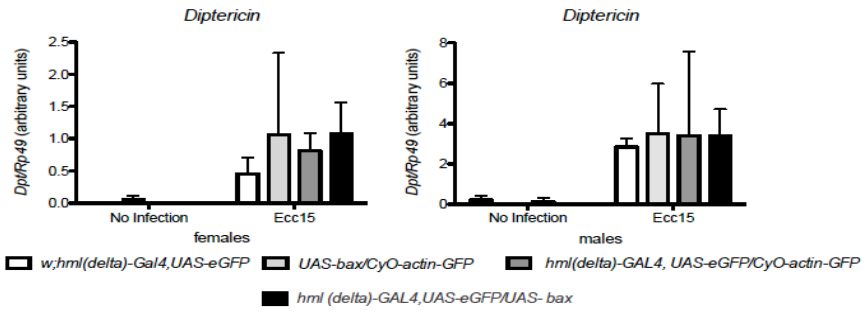
We have not converted to a log scale, as we feel the data is clear as presented.

For figure 1B, could the authors please apply ANOVA and report which transcript levels fall into different groups. This applies to all quantitation graphs.

As suggested, we applied ANOVA for Figure 1B. For all others quantitative graphs, P-values were calculated by unpaired t-test, because only two groups (control vs experimental) are compared.

I am also concerned about some of the ecdysone genes the authors are surveying and the assumption that their phenotype depends upon their effects upon antimicrobial peptide production. I'm worried about pannier and serpent in particular as both have previously been implicating in regulating another arm of the fly's immune response, namely the cellular immune response. There is a Lemaitre paper (PNAS March 28, 2000 vol. 97 no. 7 3376-3381), which looked at Erwinia induction of immune responses in flies with altered hemocytes that argued the hemocytes were required for diptericin expression. This doesn't mean that old paper is true but the result is out there. The paper raises an alternative explanation of figure 4A - that alteration of hemocyte activity reduces diptericin transcript levels.

We agree that some previous studies have suggested hemocytes have at least a partially role in the IMD-mediated synthesis of antimicrobial peptides in the larval fat body, especially during local infections. However, other reports suggest that neither larval or adult hemocytes play an essential role in this process, especially during systemic infections (Basset et al., 2000; Braun et al., 1998; Brennan et al., 2007; Charroux and Royet, 2009; Defaye et al., 2009; Dietzl et al., 2007; Matova and Anderson, 2006). To determine, in our hands with our systemic infection protocols, if the activation of the IMD pathway relies



***Phago*^{less} flies exhibit normal humoral immune response**. qRT-PCR analysis of Diptericin expression induced by *E.coli* infection in wild-type and *Phago*^{less} flies. The values represent the mean of three independent experiments and error bars represent standard deviations.

on hemocytes in adult flies, we measured the expression of *Diptericin* in upon *E. coli* infection in *Phago*^{less} flies (*HmlΔ->Bax*) (Gaumer et al., 2000; Sinenko and Mathey-Prevot, 2004). These animals lack any detectable hemocytes, as monitored by *HmlΔ->GFP,* and lack phagocytic activity. However, *Diptericin* was induced to wild type levels in these *Phago*^{less} flies upon immune challenge with *E. coli* (see figure). These data confirm the results from (Defaye et al., 2009), and argue that the *Drosophila* adult humoral IMD response to systemic infection is not particularly dependent on the presence of hemocytes. Thus, the effects observed on AMP gene induction are unlikely to be caused by the hypothetical loss of hemoctyes. It is also worth emphasizing that most of our studies use the *Yp1-GAL4* driver, that is expressed in the adult female fat body but not in hemoctyes, and hemocytes are normal in all these genotypes.

More broadly, we share the reviewers concerns about the pleiotropic effects that may be caused by interfering with these hormone signaling pathways. For this reason, our study does not rely on one approach. Firstly, we include extensive studies in S2* cells, where we monitor both the viability and morphological properties of RNAi transfected cells. In all cases, the RNAi treatments used do not cause more then ~5% cell death or notable changes in morphology during the time course of our studies, yet the IMD signaling pathway is dramatically effected, as detailed in the manuscript**.** *In vivo*, it is more challenging to control for these pleiotropic effects but we have used the Yp1 driver to avoid altering development and to focus on the fat body, the major site of systemic AMP production. Moreover, we have used RNAis targeting multiple components of the ecdysone signaling pathway, so as not to be misled by examining only one component of the pathway. Importantly, using the same Yp1-RNAi approach, we can enhance *PGRP-LC* and AMP expression, decrease susceptibility and increase bacterial clearance by targeting *Eip75B,* a known feedback negative regulator of the classic ecdysone signaling pathway. Our infection models focus on pathogens that induce an IMD-mediated response and are known to be controlled by this response (and AMPs), *i.e. Ecc15*. We also provide data with classical mutant alleles, affecting the ecdysone pathway, and new data with additional GAL4 drivers. All of these data provide, in sum, compelling evidence that ecdysone signaling modulates IMD-dependent AMP expression and function in adult flies. However, we do not claim that other aspect of the immune and defense responses are not also affected; for example the Toll pathway is also likely 20E-regulated, but will be the focus of future studies.

Referee #2 (Remarks to the Author):

Finally, depending on the AMP gene under consideration, the kinetics are totally different and here the expression of only one gene at one time point is reported.

Our approach, *in vivo*, focuses on 24 hours post infection, because at this time most AMP genes have reached their maximal levels of expression. In

other experiments, see below for one example, we have examined earlier time points and the patterns are all the same as shown in the presented data**.**

Thus, this reviewer suggests repeating in vivo some of the experiments done in cell culture, namely whether PGRP-LC ectopic expression may rescue the defective immune response observed in some 20E target gene mutants. This reviewer is aware that these experiments are difficult in that a mild overexpression of PGRP-LC is sufficient to trigger signaling independently of an immune challenge. Thus, adequate controls will be essential.

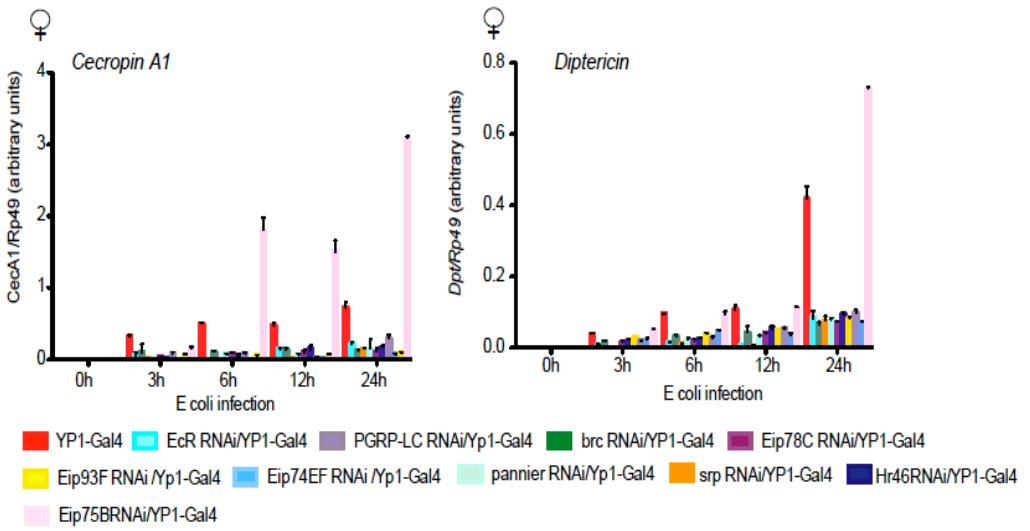
We would very much like to provide data demonstrating the *in vivo* relevance for the second mechanism of hormone control. As the reviewer points out, however, it is not trivial to perform such experiments given the effects of even mild overexpression of PGRP-LC in animals. In response to this comment/request we redoubled our efforts in this direction and used transgenic flies expressing full-length *PGRP-LCx* under the control of *YP1-GAL4* driver to drive a very high level of AMP expression, in the absence of infection. We then combined this receptor expression with the hairpin-RNAi targeting *br-c*. As predicted, depletion of *br-c* in these flies resulted in ~80% reduced expression of *Diptericin* compared to the flies expressing only PGRP-LCx. However we also found that *br-c* RNAi unexpectedly reduced the expression of our *PGRP-LCx* transgene ~50%. Therefore, these data are not easily interpretable and are not included. Due to the difficulty of creating the ideal genetic arrangement to test this part our study, *in vivo* demonstration of this second level of hormonal control will have to wait for future studies. On the other hand, both our cell-based and *in vivo* studies strongly support a role for ecdysone in the regulation of *PGRP-LC* expression.

As regards the so-called "realized" immune response, it would be important to check that the bacterial titer is indeed increasing, that is that flies are indeed succumbing to bacteremia. Also, the use of Ecc15 alone may blur the sensitivity of the assay as it may be too pathogenic. Indeed, E. coli would appear to be a much better choice. Even though some conflicting results have been published in the literature, it appears that this bacterium is unable to kill PGRP-LC mutants. Thus, if the 20E effect is solely mediated via PGRP-LC expression, one may expect that mutants for some of the 20-E target genes may be only weakly susceptible to this challenge. It would then become very meaningful to compare br-c and srp mutants against one of the Eip mutants.

As mentioned in the response to Reviewer #1, we have now provided these bacterial clearance assays for two bacteria *Ecc15* (Figure S10) and *Enterobacter cloacae* (included in this response). As predicted, interfering the ecdysone signaling pathways prevents bacterial clearance, while enhanced signaling, through *Eip75B* RNAi, enhances clearance. We have not performed similar studies with *E. coli* as *PGRP-LC* mutants are not sensitive to this non-pathogenic bacteria (Choe et al., 2002; Gottar et al., 2002; Takehana et al., 2004).

The Lemaitre 1996 Cell paper and some subsequent reports established that there were several categories of AMP genes. Interestingly, those with a short-term kinetics after an immune challenge (Attacin-A, Cecropin A and Defensin) are also those that appear to depend both on the IMD and Toll pathways when challenged with a mixture of E. coli and M. luteus. How is the kinetics of these AMP genes affected when 20E signaling is disrupted?

We are bit confused by this query. Figure 3A of the 1996 Lemaitre *et al.* paper include 1, 6, and 12 hours time points, and for wild-type flies (Or-R) the kinetics of the 6 AMP genes examined are all very similar. They are detectable at 1 hour, very robust at 6 hours with a slight increase for most at 12 hours; perhaps *Cec A1* is slightly decreased at 12 hours. In our hands with single bacteria species infections, most AMP gene expression remains high for ~24 hours post infection, and then slowly decreases. As mentioned above, the induction of AMP gene expression is very low when ecydsone signaling is blocked at several time points between 0 and 24 hours post infection (see figure below).



***EcR*, *br-c*, *Eip78C*, *Eip93F*, *Eip74EF*, *pnr*, *srp* and *Hr46* knockdown causes immunodeficiency in adult flies.** Real-time RT-PCR was used to analyze the expression of *CecA1* and *Dpt* in *EcR*, *br-c*, *Eip78C, Eip93F*, *Eip74EF, pnr*, *srp* and *Hr46* RNAi expressing flies before or at 3, 6,12 and 24 hours after infection with *E. coli*. The *yolk protein 1 (Yp1)-Gal4* driver was used to express inverted-repeat RNAs specifically in the adult female fat body, and the *Yp1-Gal4* strain is presented as a control. The mean and S.D. of three technical replicates is shown.

Minor points

1. It is interesting that the inactivation of many 20E target genes leads to a phenotype. Have the authors determined whether the expression of other 20E-

dependent genes is affected when a single gene is targeted?

Activation of the EcR/USP receptor complex triggers a complex network of interdependent transcriptional responses downstream of 20E. Several studies showed that in addition to activating 'late' ecdysone response genes, which include additional transcription factors, many of the 'early' ecdysone-inducible genes also regulate the expression of other 'early' response genes (Karim et al., 1993; Lee and Baehrecke, 2001; Thummel, 1996). For example, *br-c* was found to be required for the maximal induction of *E74EF*, *E75B* and for its own induction in late instar larvae (Karim F D, 1993).
Similarly, we found that the expression of the nine tested 20E-induced transcription factors is often affected when any single member of this group is targeted by dsRNA in S2* cells, suggesting that they regulate each other's expression in cell culture, as well.

2. The authors mention the existence of delay between hormonal signaling and PGRP-LC expression. Does this also apply to embryogenesis (Fig. S1)?

The resolution of the modEncode data is not sufficient to allow us to determine if there is a similar delay during development. Future, more exact, developmental studies will be required to examine this question.

3. *p9: when referring to dominant acting mutants, this reviewer supposes the authors mean dominant-negative?*

Yes. The text has been revised.

4. As regards the experiments with 20E target genes RNAi in S2 cells, data are only provided for AMPs that are under dual control. What happens to others?*

We also analyzed and included in the revised manuscript the expression of *Cecropin A1 (CecA1)* by qRT-PCR in in the S2* cells treated with 20E target genes RNAi and observed no induction of *CecA1* following 20E treatment and PGN stimulation (see Figure 4). Frankly, this data was inadvertently left out of the first submission.

5. If this reviewer recalls correctly, there are studies in which for instance Attacin was induced by an immune challenge in cultured cells without any 20E treatment. What is the difference due to? Different cell lines?

In our S2* cells *Attacin A* was also induced by PGN stimulation without 20E pretreatment (52-fold, on average but with a great deal of variability). However, following 24 hours hormonal pretreatment, Attacin induction was more robust (294-fold), highly reproducible ($P<0.001$), and the absolute level of expression was markedly higher (Figure 3). Given that these AMPs function as direct antimicrobial, their absolute levels of production are probably more critical then than the relative induction. Also, other studies showed that 20E treatment is required for PGN-induced enhancement of *Attacin A* expression in *Drosophila* mbn2 cells (Zhang and Palli, 2009). The requirement for ecdysone may vary

with culturing conditions, media components, or cell lines. For our cells, examining the endogenous *AttA* locus, by Northern blotting or RT-PCR, we always observe a very strong (although not absolute) dependence on hormone pretreatment.

Alternatively, have the authors attempted to stimulate their cells with heat-killed bacteria rather than purified PGN?

No, we never used heat-killed bacteria to stimulate our cells.

6. p10, last sentence: the authors refer to CecA1 data that, as noted above, are not provided in this manuscript.

We have now included the *CecA1* data for the *in vitro* experiments (Figure 4)

7. There are many plots in which statistical significance is not indicated; this appears especially important for Fig. S9 in which some error bars appear to be rather large. Which statistical procedure was used?

As suggested, we have indicated now the statistical significance throughout. The values in Figure S9 (now S6) represent the mean of three independent experiments and error bars represent standard deviations. Statistical significance was calculated by unpaired t-test.

The results shown in Fig. 3 and Fig. S5 are rather different as regards the levels of induction induced by PGN in the absence of 20E. What is this due to? Different experiments or difference due to the use of two different techniques? Have the authors checked the data yielded by qRT-PCR and nanostring analysis on the same samples?

Yes, we concur that the data in the original S5 were not as compelling, in terms of the response of *CecA1* and *AttA* especially, as compared to Figure 3. Although the patterns are similar (and most reach statistical significance), the magnitude of the response is meager. Although we have repeated the assays shown in Figure 3 numerous times (including the 3 independent assays shown in Figure 3 and others), and we are very confident of these results, the magnitude of the immune-induced AMP expression can vary from day to day. This experiment-to-experiment variation is a bit more pronounced in the stable MT-PGRP-LCx cells, possibly due to variations in the “leaky” PGRP-LC expression in the absence of copper. We do know that the weaker response presented in the original S5 is not to do the nanostring assay as compared to qRT-PCR, but is representative of the particular samples analyzed – our cells were just not responding well at the time these assays were performed. In retrospect, we erred in including this data in our first submission; it is just not representative of the vast majority of our data. In order to avoid any confusion, we have decided to exclude the nanostring data in this revised submission.

9. p5: are the references provided for the sentence on the role of PGRP-LC in

innate immunity really the most relevant ones as only one study reported some in vivo data?

As suggested we have now modified this citation to more accurately reflect the literature, including (Choe et al., 2002; Gottar et al., 2002; Kaneko et al., 2004; Kaneko et al., 2006; Leulier et al., 2003; Ramet et al., 2002; Takehana et al., 2004; Werner et al., 2003)

Referee #3 (Remarks to the Author):

The interest of this paper is based on two important results, firstly that ecdysone regulates transcription of PGRP-LC, the receptor upstream of the IMD pathway, and secondly that AMP (antimicrobial peptide gene) expression downstream of PGRP-LC is itself is under ecdysone regulation, with distinctive groups of AMPs responding more or less exclusively to ecdysone-dependent transcription factors. Taken together, the results presented in this paper clearly demonstrate the strong coupling between ecdysone-mediated transcriptional control and the IMD pathway.

Overall, we deem this study worthy of publishing, based on its solid findings on hormonal control of the immune response which extend our knowledge of this poorly characterized aspect of the immune response. However, a weakness of this study is its lack of mechanistic exploration on how ecdysone signals integrate at the level of either the PGRP-LC or different classes of AMP promoters. We feel that additional results towards these points would strengthen the paper's message and raise its suitability for a journal like EMBO.

Therefore, we list the following major recommendations:

1) Determine the mechanistic basis of ecdysone regulation on PGRP-LC and at least one of each group of AMP genes by an in-depth in silico and/or in vivo promoter analysis.

We agree that identifying which ecdysone-regulated transcription factors are directly responsible for inducing *PGRP-LC* is an important question. As we noted above, however, this hormone signaling network is highly interdependent and it is not possible to dissect a simple linear pathway leading to the regulation of PGRP-LC. On the other hand, as we mention in the text, *PGRP-LC* contains a perfect GATA element, in the third exon, that could serve as a binding site for SRP and/or PNR. A direct repeat of NHR-binding elements, which could potentially bind EcR, USP, Hr46, or Eip75B, are also found in the same exon of *PGRP-LC*. A detailed examination of these hypotheses is beyond the scope of this study but will be a focus of future experiments.

2) As the experiment shown in Figure 6 is crucial, we would recommend to reproduce it with an additional, fat body-specific driver such as c546-Gal4 (combined to a thermo-sensitive repressor Gal80 to avoid developmental effects) and verify that the drivers themselves are ecdysone-independent.

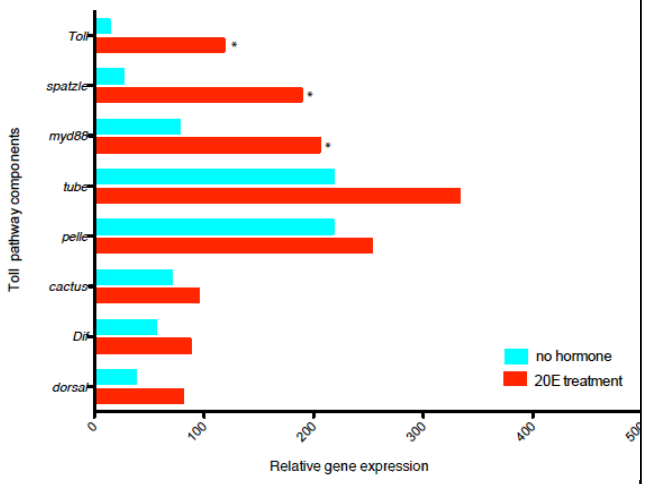
We have performed the requested experiment with the RNAi targeting

Eip78C, *Eip93F*, *Eip74EF*, *pnr*, *srp*, *Hr46* or *Eip75B* and the c564-Gal4 driver, because these animals were viable, without the Gal80 approach. We decided to avoid the Gal80 approach, because the heating regimen required to inactivate Gal80 may also affect ecdysone synthesis, or other aspect of the immune response. In particular, fly lines carrying the *Eip78C*, *Eip93F*, *Eip74EF*, *pnr*, *srp*, Hr46 and *Eip75B UAS-RNAi* constructs, as in Figure 6, were crossed with the c564-GAL4 driver, which expresses GAL4 in the adult fat body, hemocytes, as well as some male reproductive tissues (Gendrin et al., 2009) *Diptericin* expression levels in response to *E. coli* infection was significantly reduced, compared to control *c564-GAL4* driver alone animals, in all RNAi lines (except Eip75B RNAi), in both males and females (Figure S9 A-B). On the other hand, *C564-GAL4* driven *UAS-Eip75B* RNAi markedly enhanced *Dpt* expression in females, consistent with its role as a negative regulator of IMD signaling. We have similar results with a drug-inducible GeneSwitch driver and UAS-RNAi for *br-c*, *EcR* (data now shown). Of course, we additionally provide data with classical mutants affecting either ecdysone signaling or synthesis, figure S7.

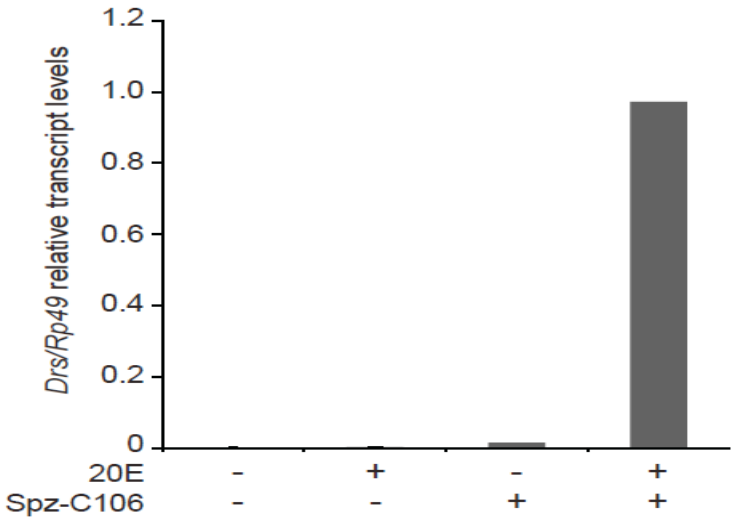
Analysing Drs expression (or any other Toll-dependent AMP) after infection with Lys-type bacteria would be an interesting control. I would also recommend to include a 3-6-12h time points with a subset of RNAi lines to test the effect of ecdysone at different time points.

The question of examining earlier time points with our RNAi experiments, *in vivo*, is addressed in the response to reviewer #2. As noted, we find that disruption of the ecdysone pathway effects *E. coli* induced AMP *induction* at all time points examined.

The ability of ecdysone signaling to modulate the Toll pathway is also an interesting question, rather then a control. In examining our microarray data, it is apparent that three Toll



20E controls Toll expression. Microarray expression profiles for Toll pathway components. Profiles were generated from triplicate samples of S2* cells before and after 24 hours of treatment with 20E. The asterisks represent statistical significance (*P value <0.05) calculated by unpaired t-test.



2**0E enhances SPZ-induced *Drosomycin* expression in S2* cells.** qRT-PCR analysis of *Drs* induction in S2* cells. Cells, with or without exposure to 20E for 24 hours were then stimulated (or not) with SPZ106 for an additional 18 hours, as indicated. (n=1) Results are representative of at least three biological replicates.

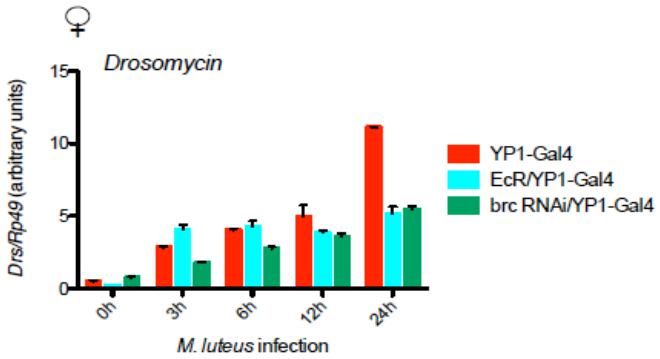
pathway components, including *Toll* itself, are significantly regulated by 20E of S2* cells (see figure previous page).

While this regulation is not 'all or none', as is the case with *PGRP-LC,* we still observe an enhanced induction of *Drosomycin* following stimulation of S2* cells with SPZC106, when S2* cells are pretreated with 20E (see figure above).

Similarly, *in vivo*, we find that disruption of 20E signaling resulted a ~2-fold reduction in *M. luteus* induced *Drosomycin* expression, at 24 hours post infection (see figure next page). However, this level of EcR-dependence is not nearly as robust as observed with IMD signaling. Therefore, the role of ecdysone in regulating the Toll pathway requires more detailed study (both in cells and in animals) and this data is provided as part of this response only, as we feel it is not yet developed for publication**.**

3) *It would be interesting to test whether ecdysone affects the IMD pathway in all tissues or whether its action is restricted to the fat body. Analyzing the local immune response in the gut of adults would answer this question.*

We agree this is an interesting question but beyond the scope of this study.



***EcR*, *br-c*, knockdown causes immunodeficiency in adult flies.** Real-time RT-PCR was used to analyze the expression of *Drs* in *EcR* and *br-c* RNAi expressing flies before or at 3, 6,12 and 24 hours after infection with *M. luteus*. *yolk protein 1 (Yp1)-Gal4* driver was used to express inverted-repeat RNAs specifically in the adult female fat body, and the *Yp1-Gal4* strain is presented as a control.

Minor suggestions

Please add statistical analysis to Fig. 4 (text mentions "mild effects" - are they non-significant?), Fig. 5, Fig. 6 (text mentions "markedly reduced" expression - significant?), Fig. S8 (text mentions "no effect" - is reduction observed with Hsf and luna non-significant?), Fig. S9 (text mentions "significantly reduced Dpt expression" - P-value?)

As suggested we have now added P-values, as appropriate throughout. All figure legends include information on the biological replicates, error bar and P-value calculations.

The normalization procedure used in Fig. 3 is unclear - are S2 cells and PGRP-LCx-FLAG cells normalized separately to their respective Dpt levels induced with 20E and PGN?*

S2* cell and PGRP-LCx-FLAG cell data were normalized separately. In each cell line, the level of each AMP in the 20E and PGN treated sample was set to 100. We have clarified in the methods section.

Since the counteracting effect of JH on 20E is mentioned in the discussion, it would add value to Fig. S1 to see also the JH peaks illustrated as bars above the graph.

We have now added the developmental JH peaks to Figure S1.

Figures S2 and S3 include stimulation with PGN for 10 min, but this is not discussed anywhere in the text. Why is this included? There is no explanation as to why 10 min exposure to PGN seems to increase PGRP-LCx expression (is PGN added to cells pulling down PGRP-LC along with the IP?)

For Figures S2 and S3 we have used the exact same samples that were used for Figure 2, because we wanted to show the levels of PGRP-LCx-FLAG for each of these four conditions/samples. For Figure 2, the 10-minute time point was chosen because that is the optimal time point for the various signal transduction events assayed(*i.e.* IMD and Relish cleavage, etc.). No PGN was added during IPs of PGRP-LC-FLAG in S3. The reason we see more PGRP-LCx (especially in the 20E treated samples) after 10 min of PGN stimulation is reproducible and might be due to ligand stabilization of the receptor, but further investigation is needed to determine if this hypothesis is correct.

However, we have now mention in the text that PGN was added to the cells 10 minutes before harvesting, and the figure legends to S2 and S3 have been clarified**.**

For readers not familiar with the nuclear receptor literature, it would be informative to add more details about the selection criteria used to choose the twelve 20E-inducible transcription factors (section 4 of Results). Likewise, the discussion mentions 64 ecydsone-inducible TFs but only 12 were analysed. Is anything known (from this study) about the 52 remaining? Are they silent with regards to IMD signalling?

Many direct targets of the EcR are themselves transcription factors, which initiate a cascade of transcriptional programs downstream of this hormone, and given the ~18 hours required to observe the effect of 20E on IMD signaling, it seemed likely that the IMD-potentiating activity is mediated by a secondary or tertiary target(s) of 20E/EcR signaling. Therefore, we analyzed our microarray data for the gene expression profiles of all known transcription factors (~700 factors) and identified 64 transcription factors that were statistically increased following 24 hours of 20E treatment. We present here an initial analysis of these transcription factors, prioritizing well-characterized “early” and “early-late” ecdysone-induced genes as well as factors previous linked to immunity (like srp). We have initial results with 17 other transcription factors, of which 10 also affect IMD signaling and/or PGRP-LC expression, while 7 do not. However, we we have not made an effort to repeat these findings multiple times and therefore they are not included in the manuscript. The remaining 35 factors are a focus of current studies. We have clarified the text, to indicate that 52 transcription factors await further study.

The observations reported in this paper are likely to explain a number of described mutations that seem to indirectly affect the IMD pathway, or the fluctuations in AMP gene expression observed in various microarrays. Optionally, this could be included in the discussion.

We are not certain as to exactly which literature the reviewer refers. However, we agree with this sentiment and we note that this has already been established for the *18wheeler* mutant (Ligoxygakis et al., 2002). Nonetheless, our text is already quite lengthy and we feel that our results speak for themselves, in terms of how the findings of others might be re-interpreted.

In case the microarray data are deposited in GEO or accessible elsewhere, please provide a link.

The microarray data will be deposited in GEO and the accession number will be provided.

Very minor corrections

All suggested corrections, below, were addressed.

Results:

1st section:

...we and others have demonstrated that the 20E modulates...

2nd section:

Occurring twice: PGRP not PRGP

...expression at its natural loci locus.

3rd section:

...the immune-induced expression of a distinct subset of AMP genes...

4th section:

"As expected, AMP genes were not induced in the parental S2 cells" - shouldn't it be "reduced"?*

5th section:

EcRNP5219, which carries a P-insertion in an EcR intron,...

At the fully restrictive temperature (29{degree sign}C), the DTS-3 mutant...

...DTS-3/+ mutant females flies... (occurs twice)

By expressing transgenic hairpin-RNAs with the Gal4/UAS system...

Please use "Erwinia carotovora carotovora 15" or Ecc15, not Erwinia carotovora

Discussion:

"RORα" not RORa

To avoid confusion, please use the same abbreviation for "Rev-Erbα" and "Eip75B" throughout (pick one - unfortunately the literature is not consistent on either gene)

...,promoting production of IL-17, Il-21 ...

...shown to trigger a systemic IMD response following a local gut infection...

"GR" define - has not come up before

...regulatory network delineated in this study is part of the neuroendocrine-immuno immuno-neuroendocrine axis...

Materials and Methods

Co-IP

Missing/erroneous symbol in "Image Reader LAS-4000"

RNAi

...stimulated with PGN for an additional 6 hours...

Confocal microscopy

...YFP-Relish and single stables cells expressing the YFP-Relish...

...35-mm glass-bottomed culture plates.

Fly strains and survival experiments

The first paper establishing the Ecc15 model of infection is: Basset A, Khush RS, Braun A, Gardan L, Boccard F, Hoffmann JA, Lemaitre B. The phytopathogenic bacteria Erwinia carotovora infects Drosophila and activates an immune response. Proc Natl Acad Sci U S A. 2000 Mar 28;97(7):3376-81.

General:

Please verify all occurrences of {degree sign}C and use the conventional {degree sign} symbol, not o in superscript

(1 occurrence in Mat&Met, 1 in Suppl Fig legends, graphs in Fig. S9)

Figure Legends

Fig.4: Eip93F missing from title - please add if omitted involuntarily

Fig.6: PGRP-LC missing from list of RNAi (as above)

Figures

Fig. 1 A: effete not effette

Suppl. Figure legends

Fig. S1: The legend says "time shown at bottom" but is displayed on top in Fig. S1.

Fig. S2: Samples were untreated or treated with 20E...

Fig. S10: EcR, Eip75B, PGRP-LC missing from title, and PGRP-LC missing from legend, please add if omitted involuntarily

...showing survival of the same males genotypes after infection...

Suppl. Figures

Fig. S3: please align - and + as in Fig. S2

Fig. S4 D: PGRP-LCx-FLAG-YFP-Relish cells

References:

Aggarwal, K., Rus, F., Vriesema-Magnuson, C., Erturk-Hasdemir, D., Paquette, N., and Silverman, N. (2008). Rudra interrupts receptor signaling complexes to negatively regulate the IMD pathway. PLoS Pathog 4, e1000120.

Basset, A., Khush, R.S., Braun, A., Gardan, L., Boccard, F., Hoffmann, J.A., and Lemaitre, B. (2000). The phytopathogenic bacteria Erwinia carotovora infects Drosophila and activates an immune response. Proc Natl Acad Sci U S A 97, 3376-3381.

Braun, A., Hoffmann, J.A., and Meister, M. (1998). Analysis of the Drosophila host defense in domino mutant larvae, which are devoid of hemocytes. Proc Natl Acad Sci U S A 95, 14337-14342.

Brennan, C.A., Delaney, J.R., Schneider, D.S., and Anderson, K.V. (2007). Psidin is required in Drosophila blood cells for both phagocytic degradation and immune activation of the fat body. Curr Biol 17, 67-72.

Charroux, B., and Royet, J. (2009). Elimination of plasmatocytes by targeted apoptosis reveals their role in multiple aspects of the Drosophila immune response. Proc Natl Acad Sci U S A 106, 9797-9802.

Choe, K.M., Werner, T., Stoven, S., Hultmark, D., and Anderson, K.V. (2002). Requirement for a peptidoglycan recognition protein (PGRP) in Relish activation and antibacterial immune responses in Drosophila. Science 296, 359-362.

Defaye, A., Evans, I., Crozatier, M., Wood, W., Lemaitre, B., and Leulier, F. (2009). Genetic ablation of Drosophila phagocytes reveals their contribution to both development and resistance to bacterial infection. J Innate Immun 1, 322-334.

Dietzl, G., Chen, D., Schnorrer, F., Su, K.C., Barinova, Y., Fellner, M., Gasser, B., Kinsey, K., Oppel, S., Scheiblauer, S., et al. (2007). A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. Nature 448, 151-156.

Gaumer, S., Guenal, I., Brun, S., Theodore, L., and Mignotte, B. (2000). Bcl-2 and Bax mammalian regulators of apoptosis are functional in Drosophila. Cell Death Differ 7, 804-814.

Gendrin, M., Welchman, D.P., Poidevin, M., Herve, M., and Lemaitre, B. (2009). Long-range activation of systemic immunity through peptidoglycan diffusion in Drosophila. PLoS Pathog 5, e1000694.

Gottar, M., Gobert, V., Michel, T., Belvin, M., Duyk, G., Hoffmann, J.A., Ferrandon, D., and Royet, J. (2002). The Drosophila immune response against

Gram-negative bacteria is mediated by a peptidoglycan recognition protein. Nature 416, 640-644.

Kaneko, T., Goldman, W.E., Mellroth, P., Steiner, H., Fukase, K., Kusumoto, S., Harley, W., Fox, A., Golenbock, D., and Silverman, N. (2004). Monomeric and polymeric gram-negative peptidoglycan but not purified LPS stimulate the Drosophila IMD pathway. Immunity 20, 637-649.

Kaneko, T., Yano, T., Aggarwal, K., Lim, J.H., Ueda, K., Oshima, Y., Peach, C., Erturk-Hasdemir, D., Goldman, W.E., Oh, B.H., et al. (2006). PGRP-LC and PGRP-LE have essential yet distinct functions in the drosophila immune response to monomeric DAP-type peptidoglycan. Nat Immunol 7, 715-723.

Karim, F.D., Guild, G.M., and Thummel, C.S. (1993). The Drosophila Broad-Complex plays a key role in controlling ecdysone-regulated gene expression at the onset of metamorphosis. Development 118, 977-988.

Kim, M., Lee, J.H., Lee, S.Y., Kim, E., and Chung, J. (2006). Caspar, a suppressor of antibacterial immunity in Drosophila. Proc Natl Acad Sci U S A 103, 16358-16363.

Lee, C.Y., and Baehrecke, E.H. (2001). Steroid regulation of autophagic programmed cell death during development. Development 128, 1443-1455.

Leulier, F., Parquet, C., Pili-Floury, S., Ryu, J.H., Caroff, M., Lee, W.J., Mengin-Lecreulx, D., and Lemaitre, B. (2003). The Drosophila immune system detects bacteria through specific peptidoglycan recognition. Nat Immunol 4, 478-484.

Ligoxygakis, P., Bulet, P., and Reichhart, J.M. (2002). Critical evaluation of the role of the Toll-like receptor 18-Wheeler in the host defense of Drosophila. EMBO Rep 3, 666-673.

Matova, N., and Anderson, K.V. (2006). Rel/NF-kappaB double mutants reveal that cellular immunity is central to Drosophila host defense. Proc Natl Acad Sci U S A 103, 16424-16429.

Ramet, M., Manfruelli, P., Pearson, A., Mathey-Prevot, B., and Ezekowitz, R.A. (2002). Functional genomic analysis of phagocytosis and identification of a Drosophila receptor for E. coli. Nature 416, 644-648.

Sinenko, S.A., and Mathey-Prevot, B. (2004). Increased expression of Drosophila tetraspanin, Tsp68C, suppresses the abnormal proliferation of ytr-deficient and Ras/Raf-activated hemocytes. Oncogene 23, 9120-9128.

Takehana, A., Yano, T., Mita, S., Kotani, A., Oshima, Y., and Kurata, S. (2004). Peptidoglycan recognition protein (PGRP)-LE and PGRP-LC act synergistically in Drosophila immunity. EMBO J 23, 4690-4700.

Thummel, C.S. (1996). Flies on steroids--Drosophila metamorphosis and the mechanisms of steroid hormone action. Trends Genet 12, 306-310.

Werner, T., Borge-Renberg, K., Mellroth, P., Steiner, H., and Hultmark, D. (2003). Functional diversity of the Drosophila PGRP-LC gene cluster in the response to lipopolysaccharide and peptidoglycan. J Biol Chem 278, 26319-26322.

Zhang, Z., and Palli, S.R. (2009). Identification of a cis-regulatory element required for 20-hydroxyecdysone enhancement of antimicrobial peptide gene expression in Drosophila melanogaster. Insect Mol Biol 18, 595-605.

2nd Editorial Decision 23 January 2013

Thank you for submitting your revised manuscript to the EMBO Journal. Your study has now been re-reviewed by the referees and their comments are provided below. As you can see the referees appreciate the introduced changes and support publication here. There are, however, a few remaining suggestions that I will outline below.

Referee #1 has no further questions

Referee #2 suggests to include the E. Cloacae data provided in the point-by-pointresponse as a supplemental figure. Since you have the data on hand I don't think it harms adding this data to the supplemental data, but I will leave that decision up to you.

Referee #3:

1. Please respond to the issue of statistical tests used.
2. The referee suggests removing the NO part from the discussion - again I will leave that up to you. The same goes for the references on the concept of resistance versus tolerance.

Once we get the revised version we will proceed with its acceptance.

Thank you for submitting your interesting study to the EMBO Journal!

———

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The authors responded answered my questions and I am satisfied with their answers.

Regarding some of the other reviewer's comments: The kinetics of AMP induction is a mess throughout the field. I haven't seen a good model as to what people think is happening. Are AMPS induced by in a PAMP dependent spike that then has a life of its own regardless of PAMP levels or is the immune response directly responding to PAMPS? There are other models. The model I have in my head is that this is simple biochemistry and PAMP levels affect induction. In this case, the timing of infection is irrelevant and all depends on microbe levels. You could look at timing but then you have to understand the differential equations that define actual antimicrobial activity, not just transcripts and how this alters microbe growth rate. I haven't seen anyone do that.

There isn't a lot of data concerning why flies die from an infection. In the cases where it is known, microbes drive wasting of the host but it doesn't necessarily take a lot of microbes to do this. It seems too much to ask the authors to work out the method of death given that nobody else does this and that it is going to be complicated as hormone signaling also regulates energy metabolism.

Concerning reviewer 3. Mechanism is fractal; by that I mean that one can always ask for another level of mechanism. My opinion is that if you can always ask for more mechanism then you should never ask for more mechanism.

Referee #2 (Remarks to the Author):

The authors have satisfactorily replied to most queries. This is a nice piece of work. This reviewer agrees with the authors' contention that the IMD pathway has mostly to do with resistance. However, the data provided in Fig. S10 are not overwhelming as little bacterial proliferation is observed (at most a Log). It would have been better to use a lower concentration of bacteria for pricking. Therefore, to make the published work stronger, this reviewer suggests including the E. cloacae data as a supplementary figure, as the data look more like what one would expect. Also, it may not be a good idea to include the 48 hours time-point. We have found that bacterial counts when flies are dying (bottom of survival curve) are rather not reliable, especially if not all flies are killed (both flies that are about to succumb and flies that have cleared the infection are sampled, the latter category being artificially increased). Therefore, a less pathogenic strain such as E. coli would have immediately provided clear-cut data (the reduction of AMP expression observed in the mutants for EcR-dependent genes seems much more pronounced than that initially reported in PGRP-LC mutants and thus should yield a survival phenotype).

Referee #3 (Remarks to the Author):

The revised paper has improved on several points (kinetics, additional driver line, bacterial counts,...). Unfortunately, a remaining weakness is the lack of mechanistical insight, to which nothing could be added during revision. It is also regrettable that the authors did not assess the impact of at least some of the RNAi lines on the local Imd response in the gut (a relatively simple experiment in our eyes). Nevertheless, we consider that this paper has the interest of deciphering the complex interaction between ecdysone and antimicrobial peptide response and could therefore be accepted for publication in EMBO.
Before releasing this manuscript for publication, we would welcome a few minor changes:

-Appropriate statistical testing (initially pointed out by all three reviewers): although the authors have now added tests and p values to all figures, they used an unpaired t test throughout, which is not the right kind of test when making multiple comparisons to the same control. The correct approach would be one-way ANOVA with post-test, which despite using a stricter significance threshold will take into account the overall scattering of data in the whole assay, and therefore improve power.

-Remove the part on NO in the discussion - recent findings (Chakrabarti S et al. Cell Host Microbe. 2012 Jul 19;12(1):60-70.) using mutants deficient in NO production showed no effect on Imd activation in the gut, hence did not recapitulate the findings of Foley et al. referenced in the text.

-Referencing 5 publications from the same lab in order to introduce the concept of resistance versus tolerance seems a bit excessive, since the corresponding experiment only appears in one supplementary figure.

2nd Revision - authors' response 02 April 2013

Point-by-Point Response to Reviewer Comments

Referee #2 (Remarks to the Author):

The authors have satisfactorily replied to most queries. This is a nice piece of work. This reviewer agrees with the authors' contention that the IMD pathway has mostly to do with resistance. However, the data provided in Fig. S10 are not overwhelming as little bacterial proliferation is observed (at most a Log). It would have been better to use a lower concentration of bacteria for pricking. Therefore, to make the published work stronger, this reviewer suggests including the E. cloacae data as a supplementary figure, as the data look more like what one would expect. Also, it may not be a good idea to include the 48 hours time-point. We have found that bacterial counts when flies are dying (bottom of survival curve) are rather not reliable, especially if not all flies are killed (both flies that are about to succumb and flies that have cleared the infection are sampled, the latter category being artificially increased). Therefore, a less pathogenic strain such as E. coli would have immediately provided clear-cut data (the reduction of AMP expression observed in the mutants for EcR-dependent genes seems much more pronounced than that initially reported in PGRP-LC mutants and thus should yield a survival phenotype).

In response to this suggestion, we have now included the *Enterobacter cloacae* data that was provided previously in the point-by-point response letter, as a supplementary figure (Figure S11) and also we increased the number of analyzed flies (15 individual flies/ time point) in order to increase the statistical power. We did not exclude the 48 hours time-point since our results show that flies depleted of *EcR*, *br-c*, *Eip78C*, *Eip93F*, *Eip74EF*, *Hr46*, *srp* or *pnr* exhibit significantly increased in *Erwinia* loads especially at 48 hours post infection, as compared to the controls, and providing both the 24 and 48 hour data gives a more complete picture. Interestingly, the phenotypes are more robust (and statistically significant) at 24 hours in the *Enterobacter* infections. This probably reflects differences in the virulence and pathogenesis of *Erwinia* (which is a rather slow and mild killer) compared to *Enterobacter*, where (as the reviewer mentions) the variance in the data becomes very large at 48 hours. This is not such an issue with the *Erwinia* infection.

Referee #3 (Remarks to the Author):

The revised paper has improved on several points (kinetics, additional driver line, bacterial counts,...). Unfortunately, a remaining weakness is the lack of mechanistical insight, to which nothing could be added during revision. It is also regrettable that the authors did not assess the impact of at least some of the RNAi lines on the local Imd response in the gut (a relatively simple experiment in our eyes). Nevertheless, we consider that this paper has the interest of deciphering the complex interaction between ecdysone and antimicrobial peptide response and could therefore be accepted for publication in EMBO.
Before releasing this manuscript for publication, we would welcome a few minor changes:

-Appropriate statistical testing (initially pointed out by all three reviewers): although the authors have now added tests and p values to all figures, they used an unpaired t test throughout, which is not the right kind of test when making multiple comparisons to the same control. The correct approach would be one-way ANOVA with post-test, which despite using a stricter significance threshold will take into account the overall scattering of data in the whole assay, and therefore improve power.

As requested we have now used ANOVA (either one-way or two-way, as appropriate) where multiple comparison are performed throughout the manuscript. In particular, statistical analysis was performed by one-way ANOVA with Tukey's Multiple Comparison Test (Figure 1B, Figure 4, 5, 6, S5, S6, S7, S8, S9) or two-way ANOVA with Bonferroni post-test (Figure S10&S11). Where only two groups were compared, unpaired t-test remain.

-Remove the part on NO in the discussion - recent findings (Chakrabarti S et al. Cell Host Microbe. 2012 Jul 19;12(1):60-70.) using mutants deficient in NO production showed no effect on Imd activation in the gut, hence did not recapitulate the findings of Foley et al. referenced in the text.

We thank the reviewer for pointing out the findings from the recent paper published by Chakrabarti S *et al*., which shows that mutation in NO synthase gene does not affect the systemic induction of AMPs upon oral bacterial infection. However, another recent paper (Shih-Cheng Wu et al., Cell Host Microbe, 2012, Apr 19; 11, 410-417) recapitulates the data, approaches and conclusions from Foley et al. , 2003 data, showing that NO triggers AMPs expression following oral infection with *Ecc15*. Our findings suggest a potential mechanism whereby NO could enhance an IMD-mediated response by interfering with E75B, therefore we decided not to remove this part from the discussion. However, we have expanded this discussion to include full citations to all these publications, and explicit mention of the lack of clarity in the field. Frankly, it remains possible that there are other chemistries to generate NO in *Drosophila* cells, beyond the enzyme encoded by the NOS gene, which is mutated in the Chakrabarti paper; this field is a bit murky, to say the least. Regardless, NO donors activate the IMD pathway and it remains possible that NO, or other related molecules which interact with the Heme group in ligand binding pocket of E75B, modulate IMD innate immunity in via this mechanism. In this section of the discussion, we mention this as a possible mechanism, rather then present a dissertation on the data supporting, or not, the exact role for NO and NOS in modulating the *Drosophila* immune response.

-Referencing 5 publications from the same lab in order to introduce the concept of resistance versus tolerance seems a bit excessive, since the corresponding experiment only appears in one supplementary figure.

As suggested we have now reduced the number of citations regarding the concept of resistance vs tolerance.