Co-option of local and systemic immune responses by the hormonal signalling system triggering metamorphosis

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ABSTRACT

Insect metamorphosis is regulated by the production, secretion and degradation of two peripheral hormones: 20-hydroxyecdysone (ecdysone) and juvenile hormone (JH). In addition to their roles in developmental regulation, increasing evidence suggests that these hormones are involved in innate immunity processes, such as phagocytosis and the induction of antimicrobial peptide (AMP) production. AMP regulation includes systemic responses and local responses, at surface epithelia that contact with the external environments.

At pupariation, *Drosophila melanogaster* increases dramatically the expression of three AMP genes: *drosomycin (drs)*, *drosomycin-like 2 (drsl2)* and *drosomycin-like 5 (drsl5)*. Using *D. melanogaster,* we show that the expression of *drs* at pupariation is dependent on ecdysone signalling in the fat body. This systemic immune response involving *drs* expression in the fat body operates via the ecdysone downstream target, Broad-Z4. In parallel, ecdysone also regulates local responses, specifically through the activation of *drsl2* expression in the gut. Finally, we establish the relevance of this ecdysone dependent AMP expression for the control of bacterial persistence by showing that flies lacking *drs* expression in the fat body have higher bacterial persistence over metamorphosis.

Together, our data establishes a new role for ecdysone during pupariation. We propose that the co-option of immune mechanisms by the hormonal cascade responsible for controlling metamorphosis constitutes a pre-emptive mechanism to control bacterial numbers in the pupa and increase developmental success.

INTRODUCTION

Insect development and physiology are mainly regulated by two peripheral hormones: the steroid hormone, 20-hydroxyecdsyone (hereafter, ecdysone) and the sesquiterpenoid hormone, juvenile hormone (JH) [1, 2]. In vertebrates, steroid hormones and their nuclear receptors are key regulators of systemic immune responses, namely through the enhancement of inflammation [3]. Similarly, in insects, steroid hormones are also known to be involved in innate immunity [4–9]. In insects with complete metamorphosis (holometabolans), AMP genes are usually silent in the absence of an immune challenge and their expression is induced upon injury and/or infection [10]. However, indirect evidence suggests that AMPs may be up-regulated at specific developmental stages irrespective of pathogen presence. Studies in the moth *Manduca sexta* show that expression of lysozyme and of the AMPs *cecropin A*, *cecropin B* and *hemolin* increases dramatically during metamorphosis, although it is not clear what regulates this increase [11–13]. This transcriptional AMP peak occurs before the end of histolysis of the larval midgut, that is, before cells are potentially exposed to any pathogen or bacterium. In this context, the accumulation of AMPs at the end of the feeding stage may constitute a response against the possible harmful effects inside the pupal body of bacteria carried-over from the gut remodelling process [14–17]. Indeed, studies in dipterans show that some species of bacteria can persist inside the host throughout metamorphosis from the larval guts [16–19]. In *Drosophila melanogaster,* up-regulation of several *drosomycin* (*drs*) paralogues has also been reported at pupariation [20]. Furthermore, five different AMPs (*diptericin, drs, attacin-A, metchnikowin* and *cecropin A1*) were shown to contain putative *cis*-regulatory elements for binding of the functional ecdysone receptor complex, suggesting an ecdysone-dependent control of their expression [21]. Importantly, this association between ecdysone signalling and immunity at this particular stage of development is not restricted to the humoral arm of the immune response. An ecdysone-dependent increase in phagocytic capacity of plasmatocytes at pupariation has been described and hypothesized to be particularly important given the higher predisposition for infection at the pupal stage [6].

In *D. melanogaster*, RNAseq analysis revealed a peak in the expression of AMPs at pupariation, of which around 95% consists of three AMPs: *drs*, *drosomycin-like 2 (drsl2)* and *drosomycin-like 5 (drsl5)* (**Fig. S1,** [20]). Considering the temporal coincidence between this phenomenon and the ecdysone peak at pupariation to regulate metamorphosis, we hypothesized that ecdysone signalling controlling development was co-opted to regulate the expression of AMPs at pupariation as a pre-emptive mechanism to control bacterial proliferation and prevent possible infections during metamorphosis associated with the gut reconstruction. We demonstrate that ecdysone regulates the expression of AMPs at pupariation, both systemically (by regulating the expression of *drs*) and locally in the midgut (by regulating the expression of *drsl2*). Importantly, the systemic expression of *drs* has an impact on the number of bacteria that persists and proliferates during metamorphosis, corroborating the hypothesis that the AMP peak has a function in controlling bacteria during metamorphosis. Furthermore, we show that this association between the expression of *drs* and ecdysone occurs solely at pupariation and through the early ecdysone-response gene Broad-Z4, which is only expressed at the end of the feeding stage [22].

RESULTS

drs, drsl2 and drsl5 are expressed at pupariation in a bacteria-independent manner

Around 95% of the AMP peak at pupariation consists of three AMPs: *drs, drsl2* and *drsl5*. Due to the gut remodelling at metamorphosis, an inefficient gut purge could lead to the activation of the canonical immunity pathways, leading to an AMP peak. To test this, we determined the expression of *drs, drsl2* and *drsl5* in mid-L3 larvae (L3) and newly pupariated (P0) pupae of germ-free (GF) or standardly-raised individuals by qPCR. We

confirmed that expression increases at pupariation for all AMPs tested (**Figure 1(A), Fig.S2(A)**), including *drs (lsmean, pairwise~{w-};+;+ | Stage*drs<0.0001), drsl2 (lsmeans(pairwise~{w-};+;+|Stage*drsl2<0.0001))* and *drsl5 (lsmeans(pairwise~{w-};+;+|Stage*drsl5<0.000))*. This increment is not lost even under GF conditions with the putative exceptions of *cecropinA1* and *defensin*. Importantly, the three main AMPs under scrutiny, *drs, drsl2* and *drsl5*, show a marked independence of bacteria presence for their expression at this transition (*lsmeans(pairwise~{w-};+;+|Stage*drs<0.0001); lsmeans(pairwise~{w-}; +;+|Stage*drsl2<0.0001); lsmeans(pairwise~{w-};+;+|Stage*drsl5<0.0001))* (**Figure 1(A),** Table S1).

Together, these results reject the hypothesis that the AMPs expression at pupariation is dependent on bacterial induction and suggest that it may be hard-wired to the developmental programme.

<u>Ecdysone regulates the systemic expression of *drs*</u>

We focused primarily on two main sources of systemic AMP expression, the fat body and haemocytes. We manipulated ecdysone signalling activity in these tissues, simultaneously and specifically, to ensure no perturbation of normal development and pupariation timing. For that we used *Cg-Gal4* combined with a *tub-Gal80[ts]*, a thermo-sensitive allele of *Gal80* under the control of the tubulin promoter (hereafter *Cg[ts]*), to drive the expression of a dominant negative ecdysone receptor under the control of the UAS sequence [23] *(EcR-A DN W650A TP3*, hereafter *EcRDN*). The reduction of ecdysone sensitivity was triggered from the onset of L3 or at wandering stage, resulting in elimination of *drs* peak at P0, compared to control conditions (**Figure 1(B)**, (*lsmeans(pairwise~Stage|Cg[ts]>mcD8GFP)=0.0001*, lsmeans(pairwise~Stage|*Cg[ts]>EcRDN)*=0.0017, lsmeans(pairwise~Genotype|P0)<0.0001)). However, the expression of *drsl2* and *drsl5* (**Figure 1(B)**, Table S2) and of the other AMPs (**Fig. S2(B)**, Table S2) were not eliminated. Furthermore, the expression of *dif*, the NF-κB factor that triggers *drs* expression upon activation of the Toll pathway, was not altered when ecdysone sensitivity was decreased (**Figure S2(C),** lsmeans(pairwise~Stage|*Cg[ts]>mcD8GFP)*=0.0162, *lsmeans(pairwise~Stage|Cg[ts]>EcRDN)=0.0015, lsmeans(pairwise~Genotype|P0)=0.3978*)**),** suggesting that the expression of *drs* at pupariation is independent of the action of the NF-κB factor.

This was further confirmed by targeting haemocytes and fat body independently, using *Hemolectin-Gal4* (*Hml-ΔGal4*) for the haemocytes and *Adipokinetic hormone receptor-Gal4* (*AkhR-Gal4)* for the fat body. When ecdysone sensitivity was reduced in the haemocytes but not in the fat body, expression of *drs*, *drsl2* and *drsl5,* as well as of other 4 AMPs, was unaffected (*lsmeans(pairwise~Stage|Hml>mcD8GFP)<0.0001*, *lsmeans(pairwise~Stage|Hml>EcR DN)<0.0001*), for the three genes), (**Fig. S3(A)** and **S3(B),** Table S3)**.** However, although reduced ecdysone sensitivity in the fat body (*AkhR>EcRDN*), did not affect the expression of *drsl2* and *drsl5* (**Fig. S3(C)**, (*lsmeans(pairwise~Stage|AkhR>mcD8GFP)<0.0001*, *lsmeans(pairwise~Stage|AkhR>EcRDN)<0.0001),* for both genes)) or of other AMPs (**Figure S3(D)**, Table S4), it resulted in decreased expression of *drs* at pupariation. (**Fig. S3(C),** *lsmeans(pairwise~Stage|AkhR>mcD8GFP) <0.0001, lsmeans(pairwise~Stage|AkhR>EcR DN) =0.6709*)).

Altogether, these results indicate that *drs* expression - but not *drsl2* or *drsl5* - is systemically regulated by ecdysone signalling specifically in the fat body at pupariation.

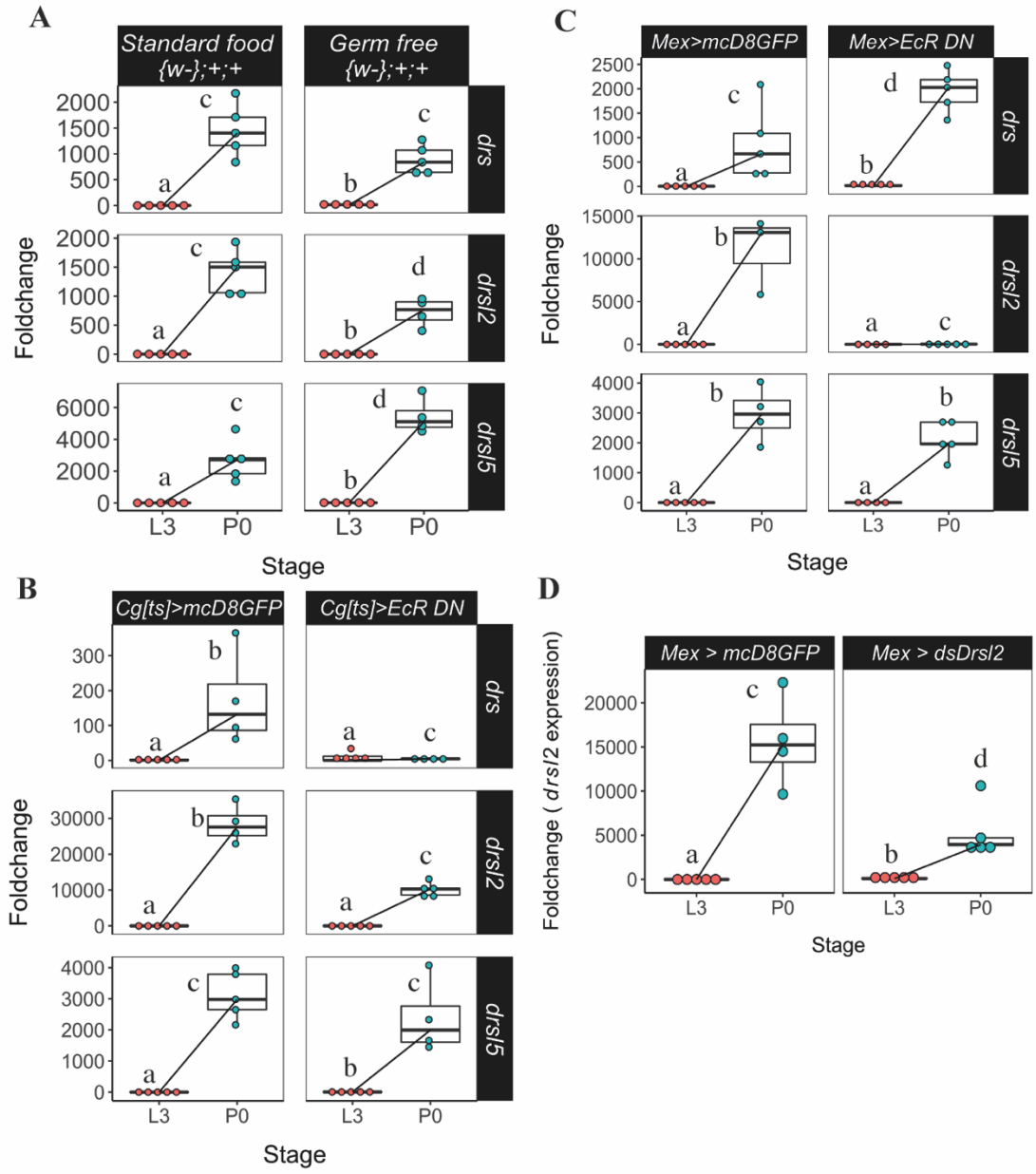

Figure 1 – Expression of AMPs at pupariation is developmentally regulated by ecdysone, both systemic and locally. (A) The expression levels of *drs*, *drsl2* and *drsl5* increase at pupariation and are not lost in GF conditions. **(B)** Foldchange increase in *drs* expression (but not of *drsl2* or *drsl5*) at pupariation was eliminated when ecdysone sensitivity is decreased specifically in the fat body and haemocytes simultaneously. **(C)** Manipulation of ecdysone sensitivity specifically in the midgut reduced the expression of *drsl2*, but not of *drs* or *drsl5*. **(D)** Knock-down of *drsl2* specifically in the midgut resulted in an overall decrease in the expression of this AMP. Each dot represents a sample of five pooled individuals; the lines connect the median of the samples at L3 and P0; different letters represent statistically significant differences in foldchange. Foldchange was determined using the ΔΔCT method.

<u>Local expression of *drsl2* in the midgut is regulated by ecdysone</u>

Having determined that the expression of these other AMPs, particularly *drsl2* and *drsl5*, are developmentally regulated, but not through the fat body, we turned our attention to two of the most important tissues for local immune response: the epithelial layers of the gut and trachea. First, we overexpressed *EcRDN* specifically in the most abundant cells of the midgut, the enterocytes (*Mex>EcRDN*). Unlike the fat body, the gut is not responding to the pupariation ecdysone pulse by increasing *drs* expression (**Figure 1(C)**). In contrast, decreased ecdysone sensitivity specifically in the midgut resulted in a drastic reduction (*circa* 600-fold) of *drsl2* expression,

very close to the L3 basal levels (**Figure 1(C)**, *lsmeans(pairwise~Genotype|P0)<0.0001; lsmeans(pairwise~Stage|Mex>EcRDN) <0.0001*)). Moreover, knock-down of *drsl2* specifically in the midgut (*MexGal4>ds-drsl2*) resulted in an overall decrease in the expression of this AMP at pupariation (**Figure 1(D)**, *lsmeans(pairwise~Genotype|P0)<0.0237; lsmeans(pairwise~Stage|Mex>ds-drsl2) <0.0001*)), which indicates that the midgut is the main source for the production of *drsl2* at this stage. Other tested AMPs also appear to be, at least partially, regulated by ecdysone signalling in the midgut. Under ecdysone-signalling down-regulation conditions, significant decrease in mRNA levels were observed for: *cecropin A1* (*lsmeans(pairwise~Genotype|P0)<0.0001; lsmeans(pairwise~Stage|Mex>EcRDN)=0.0038), defensin (lsmeans (pairwise~Genotype|P0)<0.0001; lsmeans(pairwise~Stage|Mex>EcR DN)=0.3866*), *metchnikowin* (*lsmeans(pairwise~Genotype|P0)=0.0060; lsmeans (pairwise~Stage|Mex>EcRDN<0.0001*), and *drsl3 ((lsmeans(pairwise~Genotype|P0)=0.0008; lsmeans(pairwise~Stage|Mex>EcRDN)=0.2084*), **Fig. S3(E)**)). Interestingly, no difference was detected in the expression of *drsl5*, one of the three main players in the AMP peak (**Figure 1(C),** *lsmeans (pairwise~Genotype|P0)<0.4479; lsmeans(pairwise~Stage|Mex>EcRDN)<0.0001*)).

On the other hand, decreased ecdysone sensitivity in the trachea (*btl>EcRDN*) did not have an impact on the expression of any of the three major AMPs under study (**Fig. S3(F),** Table S6).

Together, these results show that the local sensing of the ecdysone peak at the onset of pupariation in, at least the gut epithelium, controls the production of AMPs. The local and systemic mechanisms that share ecdysone control account for the expression of the genes that constitute about 70% of the pupariation AMP peak. Furthermore, the data also point towards an alternative mechanism for regulating the expression of *drsl5* at pupariation, which is either independent of ecdysone or is regulated by this hormone in an alternative tissue or cell type that were not tested.

Systemic expression of *drs* is necessary to reduce bacteria derived from imperfect larval gut purge

Before pupariation, larvae purge their gut contents to undergo metamorphosis. Any imperfection in this process creates the conditions in the pupa for a putatively rampant infection. Thus, we examined whether or not gut purge eliminates all bacteria from the gut. Under our standard rearing conditions, the mortality between P0 and pharate adult is negligible, which allows us to infer and correlate the bacterial numbers detected in each of these developmental stages (**Fig. S4(A),** Table S7).

After gut purge, approximately 5% of control P0 individuals contained bacteria in the order of the thousands and another 12% in the order of the hundreds (**Figure 2(A)**). However, at the pharate adult stage, these two classes decreased in number alongside an increase in the number of individuals without detectable bacteria (**Figure 2(B)**). In contrast, *drs* null mutants showed an increase rather than a decrease in the number of pupae with more bacteria between these two stages (**Figures 2(C)** and **2(D)**), supporting that Drs is necessary to eliminate gut derived bacteria during metamorphosis.

Using a resampling strategy, we determined that most coefficients associated with the control line were negative (**Figure 2(G)** (*Median: -2.279 (IQR: -2.682 -1.803); One-way Wilcoxon test for median<0: <2.2e-16, r= 0.864)* and **Figure 2(I)** *(Median: -2.317 (IQR: -2.907 -1.858); One-way Wilcoxon test for median<0: <2.2e-16, r=0.866)*)**.** In contrast, the coefficients associated with *drs* null mutant were mostly positive for both bacteria types (**Figure 2(H)** *((Median: 0.596 (IQR: 0.359; 0.757); One-way Wilcoxon test for median> 0: <2.2e-16, r= 0.845*) and **Figure 2(J)** (*Median: 2.055 (IQR: 1.897; 2.192); One-way Wilcoxon test for median> 0: <2.2e-16, r=0.866*)). These results suggest that pupae have the bacteria eliminated in a process that is dependent on an increase of AMPs

expression at pupariation. This process is necessary for the control the persistence of bacteria originating from gut purge imperfections, throughout metamorphosis.

When ecdysone signalling activity is decreased in the fat body and haemocytes, a similar pattern can be observed: there is a higher proportion of individuals with larger loads of persisting bacteria (**Figure 2(E-F)**). The resampling strategy revealed that *Cg[ts]>EcR DN* samples have positive slope values for both *Lactobacillus* (**Figure 2(K)**, *Median: 2.746 (IQR: 2.562; 2.930); One-way Wilcoxon test for median>0: <2.2e-16, r=0.866)* and *Acetobacter* (**Figure 2(L)**, *Median: 1.981 (IQR: 1.795; 2.184); One-way Wilcoxon test for median>0: <2.2e-16, r=0.866)*, which is in accordance with increased bacterial loads during metamorphosis. Importantly, *Mex>EcRDN* individuals do not have increased bacterial loads during metamorphosis, suggesting that the local immune response does not have a measurable antibacterial effect relatively to the systemic response that is still operating in these conditions (**Fig. S3(B-E)**).

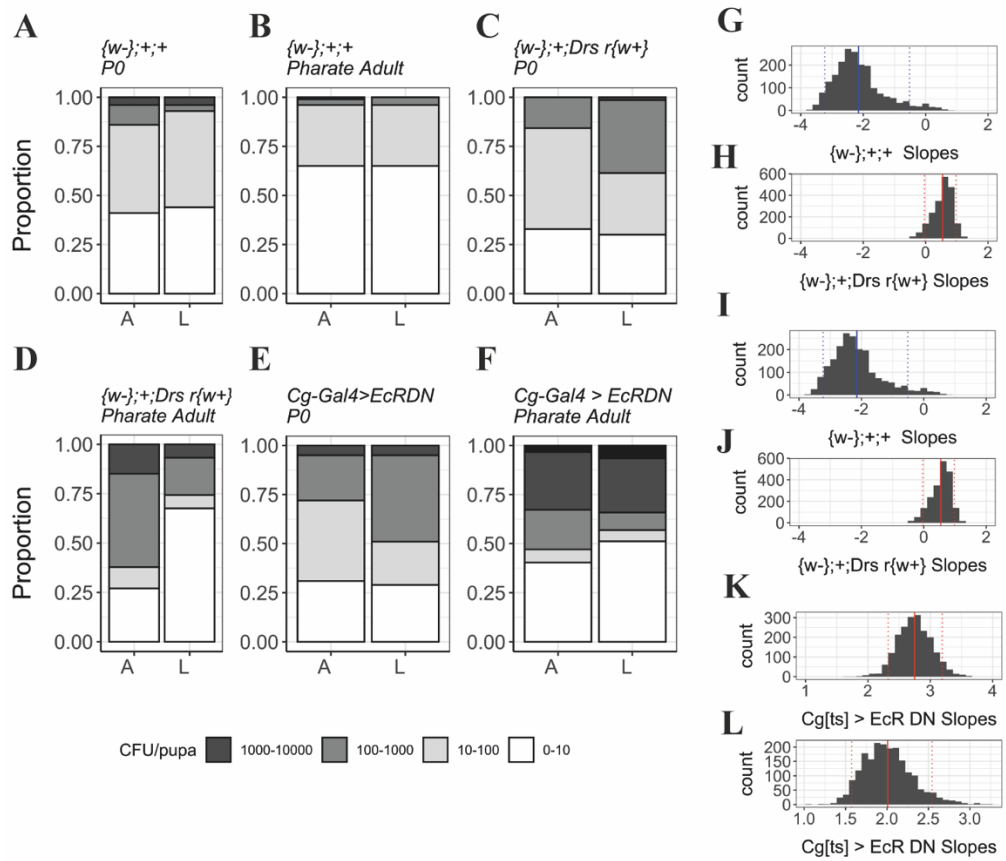

Figure 2 – Expression of AMPs at pupariation is developmentally regulated by ecdysone, both systemic and locally. **(A-F)** Quantification in P0 pupae and pharate adults of *Lactobacillus* (L), growing in selective MRS medium, and *Acetobacter* (A), growing on selective D-Mannitol. **(A-B)** In control flies (*{w-};+;+*)**,** the number of pupae with more bacteria decreases between P0 and pharate adult stages. **(C-D)** In the *drs* mutant line (*{w-};+; Drs r{w+}*), there are more individuals with higher loads of bacteria persisting from P0 to pharate adult. **(E-F)** When ecdysone sensitivity is decreased in fat body and haemocytes, the number of both bacteria types increases in P0 and pharate adults. Y-axis represents the proportion of individuals in each bacterial count category. **(G-H)** Slopes for the number of *Lactobacillus* in control and the mutant lines. **(G)** Control samples have negative slopes, representative of a decrease in the number of bacteria between stages. **(H)** *drs* mutant samples have positive slope values, representative of an increase in the number of bacteria between the stages. **(I-J)** Slopes for the number of *Acetobacter* in the control and the mutant lines. **(I)** Control samples distribution show negative slopes, i.e., a decrease in the number of bacteria between stages. **(J)** *drs* null mutant showed a positive slope values. **(K-L)** Coefficient (slopes) distribution in *Cg[ts]>EcRDN* samples of *Lactobacillus* (**K**) and *Acetobacter***.** Doted lines represent the 0.05 and 0.95 quantiles; full line represents the mean slope**.**

198 Br is involved in systemic *drs* induction through the fat body at pupariation

199 Our data suggests that the systemic expression of Drs is more important than the local production of AMPs
200 for the control of bacterial persistence during metamorphosis. Hence, we focused our efforts in trying to uncover the
201 molecular regulation of temporal *drs* expression.

202 Previous RNAseq data showed that the expression of *drs* markedly increases at pupariation (**Figure S1,**
203 [20]). We further confirmed that the expression of *dhr3* (a gene directly regulated by the ecdysone-ecdysone
204 receptor complex) and that of *drs* are tightly correlated and peak exclusively at pupariation (**Figure S5(A)**).
205 Importantly, this robust expression of *drs* at the onset of metamorphosis seems to be a general feature regardless of
206 their genetic background (**Figure S5(B)**).

207 *br* is a direct target of ecdysone receptor, characterized as a holometabolan pupal specifier with increased
208 expression before pupariation [22, 24, 25]. We examined the association between *br* and the ecdysone-sensitive
209 systemic induction of *drs* at metamorphosis by knocking-down *br* specifically in the fat body and haemocytes. This
210 led to a significant decrease in the expression of *drs* at pupariation, when compared to the control (**Figure 3(A),**
211 *lsmeans (pairwise~Genotype|P0)=0.0510*)), suggesting a role for *br* in the regulation of AMP expression. To further
212 explore this possibility, we simultaneously reduced ecdysone signalling and restored *br* in the haemocytes and fat
213 body. This was sufficient to rescue the expression of *drs* to control levels at P0 (**Figure 3(B),**
214 *lsmeans(pairwise~Genotype|P0)= 0.3068*). Furthermore, we generated two different conditional CRISPR-mediated
215 knock-out constructs [26, 27], one targeting a common region to all *br* isoforms and, the other, a region specific to
216 *br-Z4,* previously associated with AMP expression in a different species [28]. Knock-out of all *br* isoforms or of *br-*
217 *Z4* alone in haemocytes and fat body, led to the elimination of the *drs* peak at pupariation (**Figure 3(C),** *lsmeans*
218 *(pairwise~Stage|Cg>+;Cas9;+)<0.0001;lsmeans(pairwise~Stage| Cg>Cas9;br KO)=0.0001,*
219 *lsmeans(pairwise~Stage|Cg>Cas9;br-Z4 KO)=0.4691*), supporting that Br is involved in the regulation of *drs*
220 expression at pupariation downstream of the ecdysone receptor complex.

221 To further explore this regulation of *drs* expression by Br-Z4, we used the JASPAR
222 (http://jaspar.genereg.net) matrix MA0013.1 to run a comprehensive search for Br-Z4 binding sites over 2 kb of
223 putative promoter region that is upstream of the coding sequence of *drs*, obtained from Flybase (http://flybase.net/),
224 using FIMO software [29] (**Fig. S5(C)**). We identified three putative Br-Z4 binding sites (p-value<0.0001) in the
225 predicted 5' promoter region of *drs* (**Figure S5(D)**).

226 Together, these results support the idea that Br, specifically isoform Br-Z4, is involved in the regulation of
227 the expression of *drs* at pupariation.

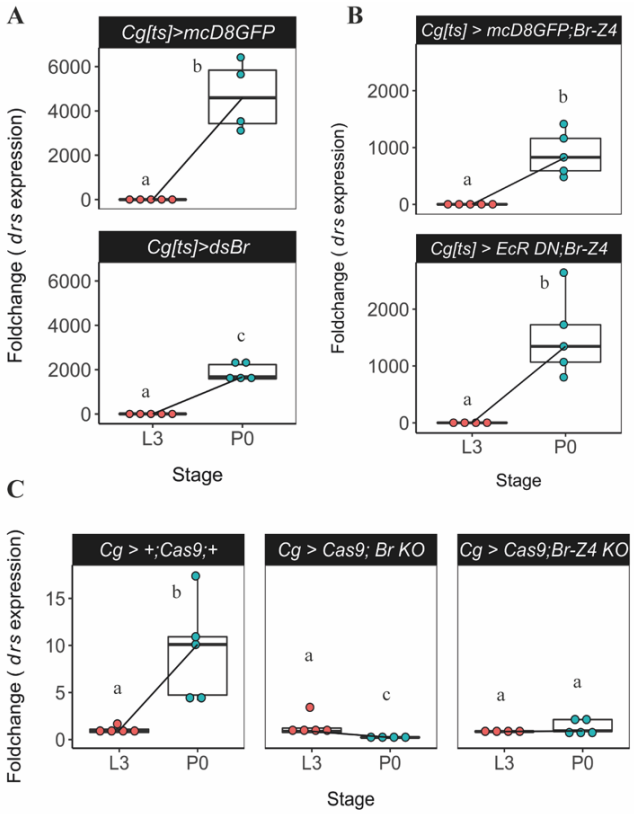

Figure 3 – Ecdysone signalling regulates fat body expression of *drs* at pupariation via Br-Z4. (A) Knockdown of *br* specifically in the fat body and haemocytes leads to decreased expression of *drs* at P0. (**B**) Co-overexpression of *br-Z4* when ecdysone sensitivity is decreased in the fat body and haemocytes is sufficient to rescue the expression of *drs* at pupariation (**C**) Conditional CRISRP-mediated knock-outs for the common region of *br* and *br-Z4* lead to loss of *drs* expression at pupariation. Each dot represents a sample of five pooled individuals; the lines connect the median of the samples at L3 and P0; different letters represent statistically significant differences in foldchange, determined using the ΔΔCT method.

DISCUSSION

Generally, in Holometabola, the expression of AMPs is induced by sensing of specific molecular patterns that trigger the activation of the Toll or Imd pathways [10, 30–33]. However, there is increasing evidence of alternative regulators of innate immunity, including the moulting hormone, ecdysone [4–9]. In vertebrates, it has been known for many years that the immune system influences the secretion of hormones and that these, in turn, can influence immunity [3].

In this study we explored the association between ecdysone and the increased expression of *drs* and its paralogues at pupariation. We unravelled a novel association between the endocrine and immune systems in *D. melanogaster*, which act both systemically and locally during metamorphosis. Importantly, we could determine that the systemic expression of *drs* has an effect on the number of bacteria during metamorphosis, as both mutant and ecdysone-reduced sensitivity individuals showed an increase rather than a decrease in the number of bacteria between P0 and pharate adult stages. Interestingly the effect was detected for both Gram-positive (*Lactobacillus*) and Gram-negative (*Acetobacter*) bacteria. Although Drs is generally characterized as an antifungal AMP, previous studies already demonstrated that it can also be induced by both Gram-positive and Gram-negative bacteria [33–39]. As such, the unexpected higher impact of the lack of *drs* on *Acetobacter* could actually be explained by its possible direct impact on Gram-negative bacteria. Importantly, the humoral arm of defence is not the only one acting at this stage of development. Regan and colleagues demonstrated that at pupariation, ecdysone is required to activate

254 haemocytes and to orchestrate the active migration of these cells towards wounding sites [6]. Therefore, at this stage
255 both ecdysone-regulated cellular and humoral mechanisms act in synergy to decrease the probability of infection.
256 Because the lack of local expression of *drsl2* did not affect bacterial persistence *per se* in our conditions,
257 we focused our efforts on trying to disentangle the molecular regulation of *drs* expression in the fat body. We
258 showed that the expression of *drs* at this stage depends upon the expression in the fat body of the ecdysone
259 signalling target, *br-Z4*. This is in accordance to previous indications that Br is essential for the PGRP-LC-
260 independent hormonal regulation of *drs* [4]. Furthermore, similar ecdysone-mediated immune activation through Br
261 has been previously observed. Studies in the silkworm *B. mori* showed that during metamorphosis, ecdysone
262 activates the transcription of lysozyme via Br-Z2 [40] and of the AMP *lebocin* via Br-Z4 [28]. This regulatory
263 mechanism through Br may cause the stage specificity. Although ecdysone controls all the moults throughout
264 development, *drs* expression increases in response to this hormone specifically at pupariation, when Br is expressed
265 in Holometabola [22, 41]. The regulation of AMPs by Br is an interesting feature, as it can also have implications on
266 the interpretation of AMP expression throughout development between holo- and hemimetabolan insects. Johnston
267 [42] demonstrated that these two insect groups have different AMP expression patterns, with holometabolans
268 showing a strong upregulation of immunity that coincides with metamorphosis timing and Br expression. In
269 contrast, hemimetabolans do not undergo a dramatic increase in immunity, possibly correlating with much less
270 drastic changes in gut morphology during progressive metamorphosis. In accordance, there is indication that insects
271 with complete metamorphosis suffer a reduction in density, diversity and altered composition of bacterial
272 populations during metamorphosis [43–45], whereas those with incomplete metamorphosis show a constant increase
273 in microbial density and diversity throughout development [46]. These data, as ours, are consistent with the
274 previously proposed hypothesis that complete metamorphosis elicits a prophylactic immune response [12, 13].
275 Alterations in immunity at life stages with higher risk of infection seem to be a general feature of
276 development, finding some parallels in both vertebrates and invertebrates. In *D. triauraria*, genes of the *drs* family
277 are induced during diapause, which is also considered to be a more susceptible period [47]. Most interestingly, data
278 from *Xenopus* also point towards an association between the timing of metamorphosis and a shift in the immune
279 response [48]. For example, Major Histocompatibility Complex I (MHC class I) molecules, which bind to peptide
280 fragments derived from pathogens, are first detected at the beginning of metamorphosis [49] and the expression of
281 MHC class II molecules is not triggered if metamorphosis is chemically blocked [50].
282 Together, our data suggests a regulatory mechanism through which ecdysone co-opted the immune system
283 to enhance the success of metamorphic processes. This study reveals a novel association between the endocrine and
284 immune systems in *D. melanogaster*, which act both systemically and locally during metamorphosis. This
285 recruitment of immunity mechanisms by the holometabolan metamorphosis programme presents other interesting
286 features. Three AMPs explain around 95% of the peak observed at the L3 to pupal transition. Interestingly, these
287 three AMPs have been co-opted differently and complementarily to produce, what we interpret as a full-fledged
288 immune prophylactic response. One of them, *drsl2*, was recruited to one of the main exposed surface epithelia of
289 larva, the midgut, that undergoes extensive remodelling at metamorphosis. Another of the AMPs with increased
290 expression, *drs*, is produced in the fat body at metamorphosis initiation, executing a putatively systemic action. The
291 local and systemic mechanisms that share ecdysone control and that were explored in this work account for the
292 expression of the genes that constitute close 70% of the pupariation AMP peak.
293 This specificity and multi-layered developmentally-regulated immune response underscores the sophistication of
294 the co-option process and hints as a possible contribution of this response to the success of metamorphosis as a whole.

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411
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414

415 **MATERIALS AND METHODS**

416

417 *Drosophila* Stocks and Husbandry

418 If not further noticed, *w[1118]* flies were obtained from Dr. Luís Teixeira (Instituto Gulbenkian de Ciência,
419 Portugal) and used as a wild-type strain. The origin of other fly strains is mentioned in SI. All fly lines we kept at
420 25ºC in *Drosophila* cornmeal food (Supplementary Information), in a 12L:12D photoperiod. For the ecdysone
421 decreased sensitivity experiment and tissue specific CRISPR, animals were maintained at 17ºC until the moult to the
422 L3 and then transferred to 29ºC to activate Gal4 activity.

423

424 CRISPR Knock-out Flies Generation

425 The tissue-specific UAS *br* CRISPR knockout constructs were cloned into pCFD6 vector (www.crisprflydesign.org,
426 Addgene #73915) according to manufacturer's instructions. Four independent gRNA constructs were cloned to
427 target introns (see primers listed in **Table S9**) of the *br* core region genomic DNA sequence for *br* common
428 construct and Z4 isoform specific sequence for *br-Z4* construct to target only native genes but not transgenes. After
429 sequenced, the constructs were inserted on either the second chromosome in a recipient line carrying phiC31
430 integrase and attP40 landing cite with an in-house modified line (*y,w, P{y+.nos-int.NLS}; P{CaryP y+}attP40*) or
431 the third chromosome in a recipient line carrying phiC31 integrase and attP2 landing site, *y,w, P(y[+].nos-int. NLS);*
432 *P(CaryP)attP2* (gift from Dr. Diogo Manoel, Sidra Medical and Research Center, Qatar). Transgenesis was carried
433 out in-house at the Instituto Gulbenkian de Ciência, Portugal.

434

435 Larval and pupal staging

436 Larvae were resynchronized every 2h and kept in normal food for the appropriate time before collection in Trizol.
437 White pre-pupae (P0) were collected directly in Trizol every 20 minutes. All samples were kept at -80ºC until use.
438 More details are available as SI.

439

440 P0 and adult pharate collection and platting

441 P0 samples were collected as aforementioned. Pharate adults were also collected in 20 minutes intervals 48h to 72h
442 after P0 collection and were identified as fully developed adults still to eclose. All samples were surface sterilised
443 before platting onto MRS or D-Mannitol plates. Plates were incubated at 25ºC for 48h. More details as SI.

444

445 Statistical analysis for bacterial growth across stages

446 Growth across stages was assessed with the generation of simulated data from real data. The initial sample was
447 resampled in subsets of 50 entries for 2000 cycles. In each cycle, a negative binomial regression was performed to
448 model the change of bacteria according to the stage, materialized in a regression coefficient, or slope. These slopes
449 were then analysed to see the central tendency of the population. In parallel, the effect size was also calculated.
450 More details on statistical analysis on SI.

451

452 Germ-free fly generation

453 Newly laid eggs were collected into a sterile embryo basket and washed with autoclaved MilliQ water. The eggs
454 were dechorionated, sterilised and transferred to axenic media supplemented with antibiotics. Plates and/or bottles
455 with embryos were kept in a 25ºC inside sterilized containers at 25ºC. After the treatment, the germ-free status was

validated by plating surface-sterilized adult flies (from both germ free and control conditions) onto MRS and D-Mannitol media.

RNA extraction, cDNA synthesis and real-quantitative PCR

RNA was isolated using the Direct-zol™ RNA MiniPrep Kit (Zymo®), following manufacturer's instructions. DNase I treatment and cDNA synthesis were preformed using the RQ1 RNASE-FREE DNASE 1* (Promega®) and the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific®), respectively. qPCR was performed using the SYBR® Green PCR Master Mix (Applied Biosystems®) and ABI 7900HT (Applied Biosystems). More details on PCR conditions and data analysis on Supplementary Information.

Br binding sites prediction

The DNA binding motif matrix for Br-Z4 was obtained from the JASPAR database (http://jaspar.genereg.net) and the 5' 2kb region of the *drosomycin* gene was acquired in Flybase (http://flybase.org). Both sequences were uploaded in FIMO software (http://meme-suite.org/tools/fimo) [29], using a p-value < 0.0001 as a filter.

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Contributions

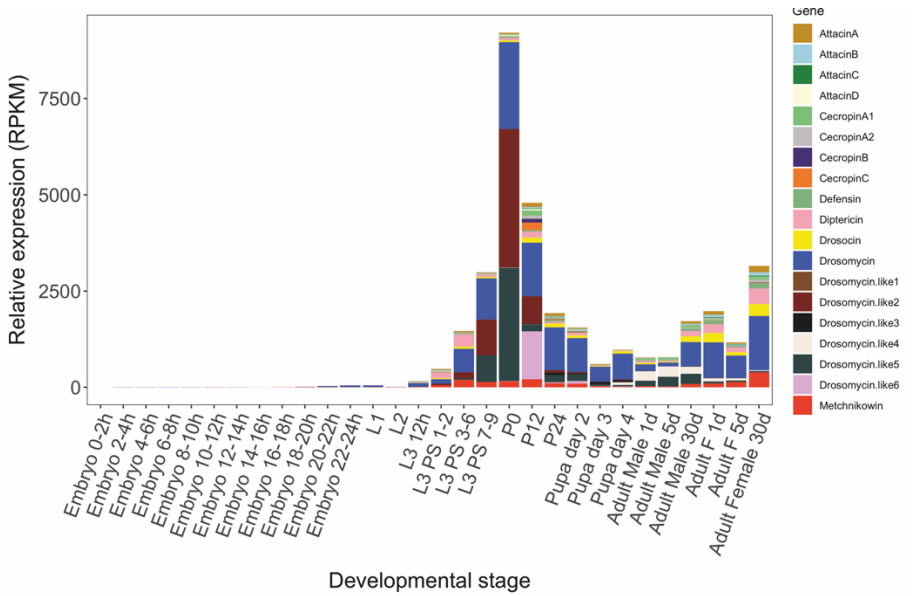
CN, Conceptualization, Formal analysis, Investigation, Visualization, Methodology, Writing—original draft, review and editing

TK, Conceptualization, Investigation, Methodology, Writing—review and editing

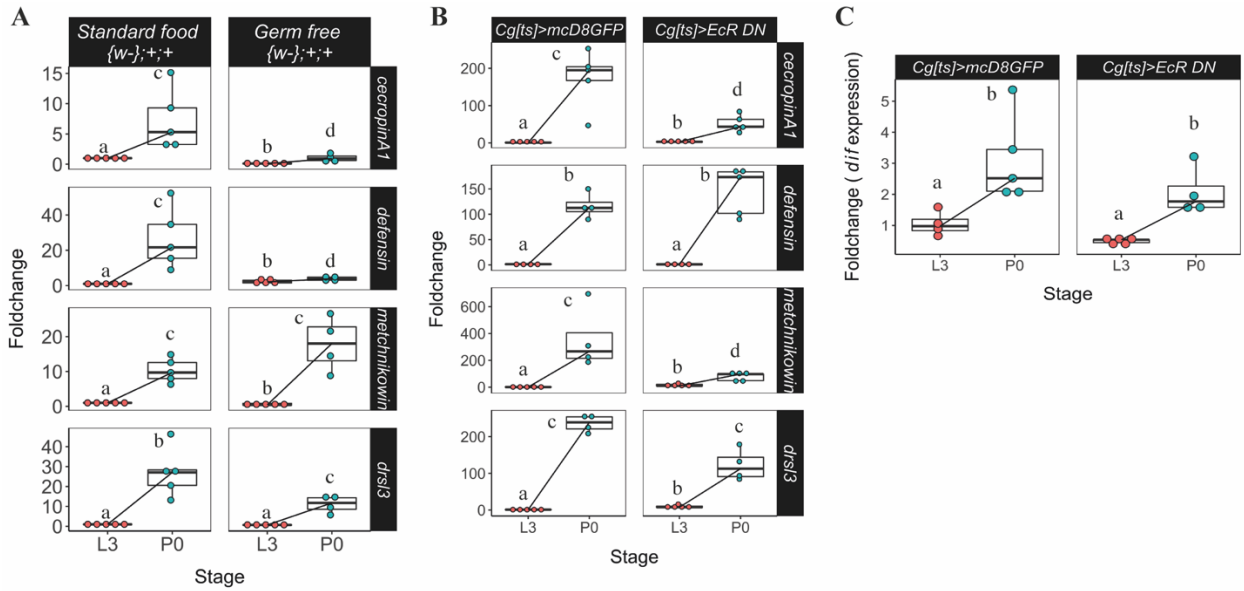
ES, Conceptualization, Investigation, Funding acquisition, Methodology, Writing— original draft, review and editing

SUPPLEMENTARY INFORMATION

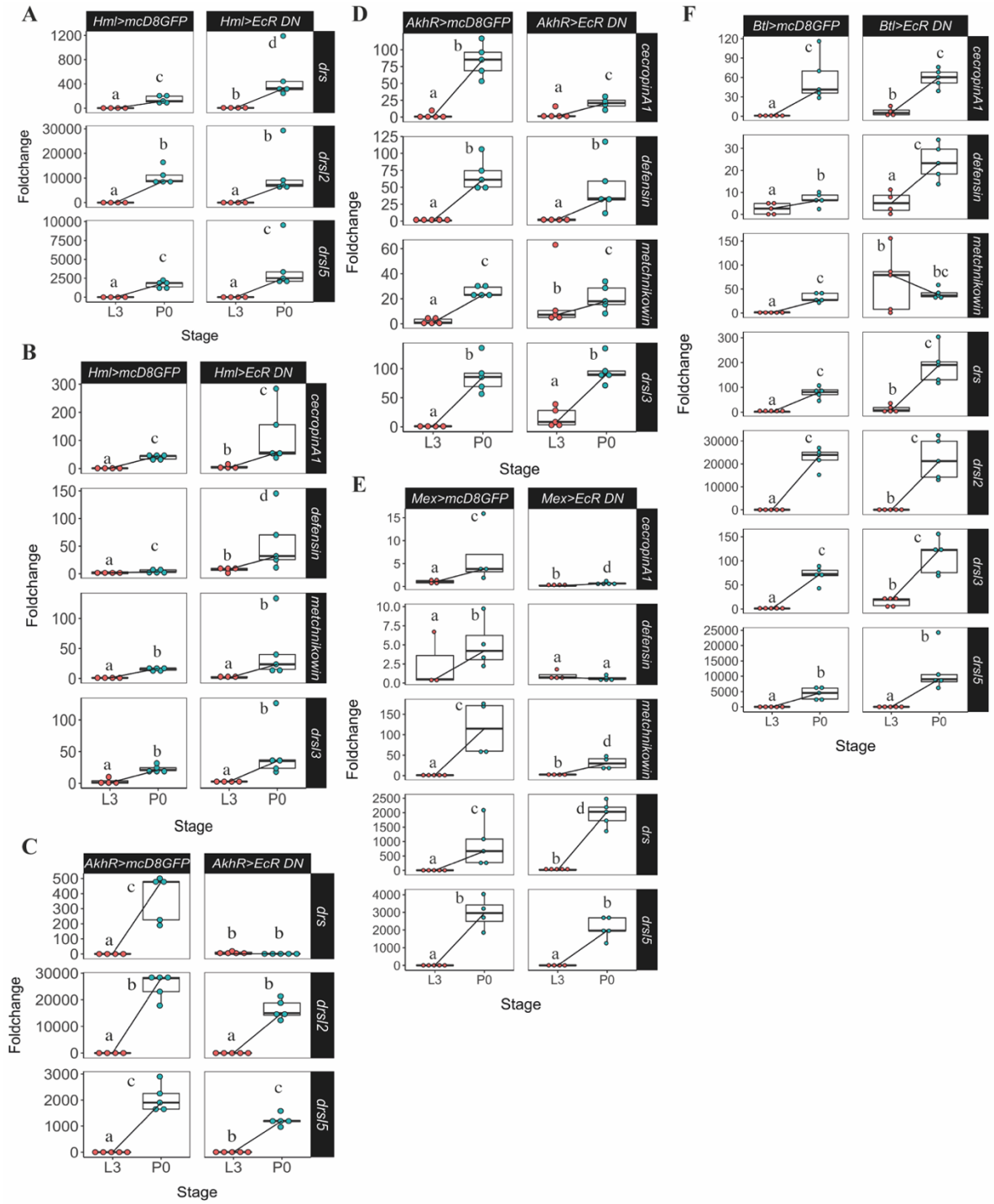
Supplementary Figure S1 – High-throughput temporal expression data using whole body, with expression levels represented as linear and scaled to maximum expression (publicly available on Flybase http://flybase.org/ [20]).



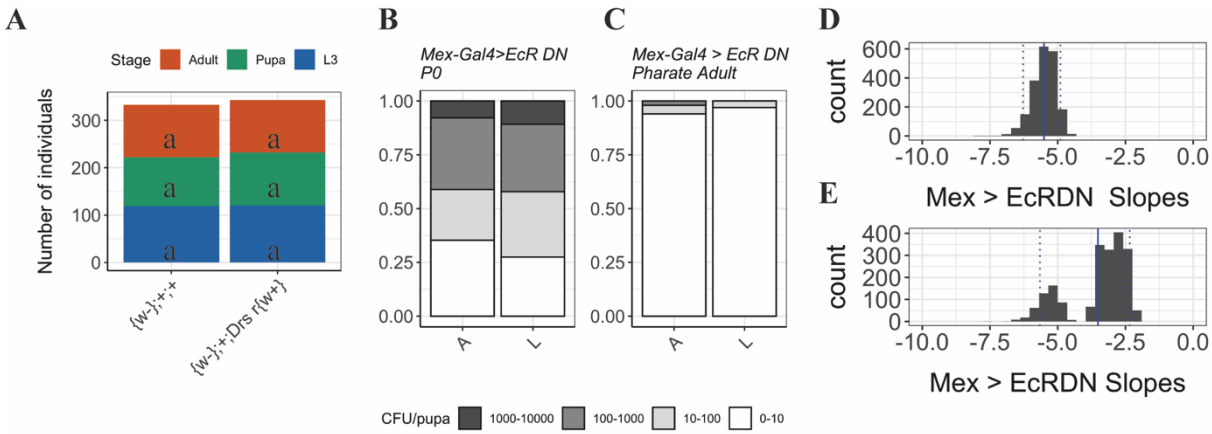
Supplementary Figure S2 - (A) The expression levels of *cecropin A1, defensin, metchnikowin* and *drsl3* increase at pupariation and are not lost in GF conditions, albeit to different degrees. **(B)** Reduced expression of ecdysone sensitivity in the fa body and haemocytes does not affect the expression of *cecropin A1, defensin, metchnikowin* and *drsl3*. **(C)** *dif expression* was not affected by the decreased ecdysone sensitivity in the haemocytes and fat body (*lsmeans(pairwise~Genotype|P0)=0.3878*). Each dot represents a sample of five pooled individuals; the lines connect the median of the samples at L3 and P0; different letters represent statistically significant differences in foldchange. Foldchange was determined using the ΔΔCT method.



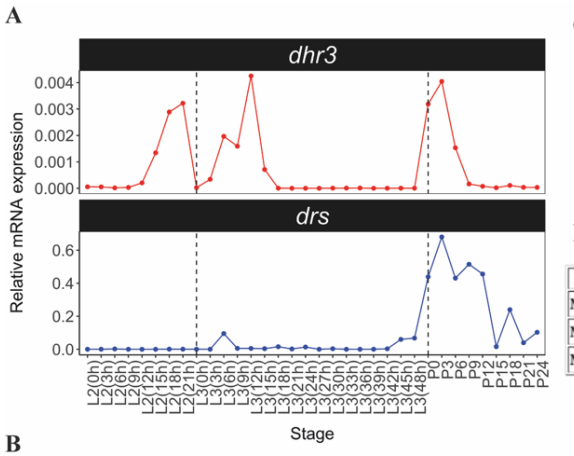
Supplementary Figure S3 – (A) Reduced ecdysone sensitivity in the haemocytes does not affect the expression of the AMPs under study. **(B)** Expression of other 4 AMPs was also not reduced when ecdysone signalling was reduced in the haemocytes. **(C)** Reduced ecdysone signalling in the fat body resulted in decreased *drs* expression at pupariation (*lsmeans(pairwise~Stage|AkhR>mcD8GFP)<0.0001, lsmeans(pairwise~Stage|AkhR>EcR DN) =0.6709*), without affecting the expression of *drsl2* and *drsl5* (*lsmeans(pairwise~Stage|AkhR>mcD8GFP)<0.0001*, *lsmeans(pairwise~Stage|AkhR>EcRDN)<0.0001)*, for both genes). **(D)** The expression of *cecropin A1*, *defensin*, *metchnikowin* and *drsl3* was unaffected by decreased ecdysone sensitivity specifically in the fat body. **(E)** Decreased ecdysone signalling in the midgut resulted in significant decrease in mRNA levels for: *cecropin A1* (*lsmeans(pairwise~Genotype|P0)<0.0001; lsmeans(pairwise~Stage|Mex>EcRDN)=0.0038), defensin (lsmeans (pairwise~Genotype|P0)<0.0001; lsmeans(pairwise~Stage|Mex>EcR DN)=0.3866*), *metchnikowin* (*lsmeans(pairwise~Genotype|P0)=0.0060; lsmeans (pairwise~Stage|Mex>EcRDN<0.0001*), and *drsl3* (*(lsmeans(pairwise~Genotype|P0)=0.0008; lsmeans(pairwise~Stage|Mex>EcRDN)=0.2084*). **(F)** Ecdysone signalling in the trachea does not affect the expression of any AMP under study.

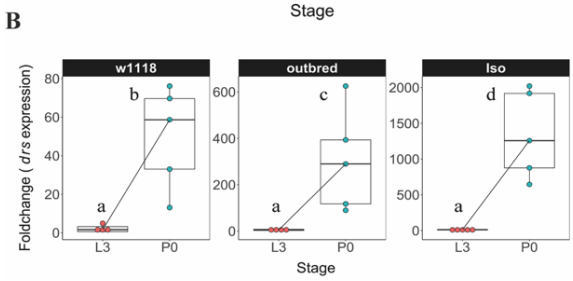


Supplementary Figure S4 – (A) Control ({*w-*};+;+) and *drs* mutant ({*w-*};+; *Drs r*{*w+*}) lines showed no differences in mortality between L3, pupae and adults (*lsmeans(pairwise~Stage|Genotype)<0.0001; lsmeans(pairwise~Genotype|Stage)<0.0001*). **(B-C)** Quantification in P0 pupae and pharate adults of *Lactobacillus* (L), growing in selective MRS medium, and *Acetobacter* (A), growing on selective D-Mannitol. The number of individuals with more bacteria is greater in P0 samples (**B**) than in pharate adults **(C)**. Y-axis represents the proportion of individuals in each bacterial count category. **(D-E)** Coefficient (slopes) distribution in *Mex>EcRDN* samples of *Lactobacillus* (**D**) (*Median: -5.455 (IQR: -6.279; -4.895); One-way Wilcoxon test for median<0: <2.2e-16, r=0.866*) and *Acetobacter* **(E)** (*Median: -3.134 (IQR: -5.656; -2.342); One-way Wilcoxon test for median>0: <2.2e-16, r=0.866)*. Doted lines represent the 0.05 and 0.95 quantiles; full line represents the mean slope.



Supplementary Figure S5 - (A) *drs* expression was temporally correlated with a proxy of ecdysone activity, *dhr3*. Samples of *D. melanogaster* larvae and pupae were precisely staged at the onset of the instars and collected every 3h. The expression of *dhr3* and *drs* was determined by qPCR for each timepoint. Dashed black lines represent the moult to the L3 or pupariation. **(B)** The expression of *drs* increased at pupariation regardless of the genetic background (*lsmeans(pairwise~Stage|w1118)<0.0001; lsmeans(pairwise~Stage|outbred<0.0001; lsmeans(pairwise~Stage|Iso)<0.0001*). Each dot represents a sample of five pooled individuals; the lines connect the median of the samples at L3 and P0; different letters represent statistically significant differences in fold-change. Fold-change was determined using the ΔΔCT method. **(C)** JASPAR DNA binding motif MA0013.1 for Br-Z4. **(D)** Br-Z4 binding motifs predicted by FIMO [29] software (http://meme-suite.org/tools/fimo) to be present in 2kb region of the 5'-end of the *drs* gene, using a p-value<0.0001 as filter.



D

Motif ID	Alt ID	Sequence Name	Strand	Start	End	p-value	q-value	Matched Sequence
MA0013.1	br(var.4)	Drs	+	1022	1032	8.65e-06	0.0254	AAGTAAACAAA
MA0013.1	br(var.4)	Drs	-	1189	1199	1.9e-05	0.0279	TAATAAACAAC
MA0013.1	br(var.4)	Drs	+	755	765	7.17e-05	0.0703	TAGAAAAGAAA