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1 **Recognition of non-self is necessary to activate *Drosophila*'s**
2 **immune response against an insect parasite**
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20
21 ## Abstract
22

23 Innate immune responses can be activated by pathogen-associated molecular
24 patterns (PAMPs) or danger signals released by damaged tissues. As PAMPs are
25 typically conserved across broad groups of pathogens but absent from the host,
26 it is unclear whether they allow hosts to recognize parasites that are
27 phylogenetically related to themselves, such as parasitoid wasps infecting
28 insects. Parasitoids must penetrate the cuticle of *Drosophila* larvae to inject their
29 eggs. In line with previous results, we find that the danger signal of wounding
30 triggers the differentiation of specialized immune cells called lamellocytes.
31 However, using oil droplets to mimic infection by a parasitoid wasp egg, we find
32 that the activation of melanization response that kills parasitoids also requires
33 exposure to a parasitoid wasp molecule that acts as a PAMP. The unidentified
34 factor enhances the transcriptional response in hemocytes and induces a specific
35 response in the fat body that includes *Tep1*, which is essential for efficient
36 melanization. We conclude that a combination of danger signals and PAMPs are
37 required activate *Drosophila*'s immune response against parasitic insects.
38

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39
40 ## Introduction
41
42 Organisms must be able to reliably detect when they are infected in order to
43 mount an appropriate immune response, and this frequently relies on the
44 recognition of non-self. In the case of innate immune systems, pattern
45 recognition receptors (PRRs) detect pathogen-associated molecular patterns
46 (PAMPs). These are typically molecules such as flagellin or peptidoglycan that
47 are absent from the host but highly conserved across a broad class of pathogens
48 [1]. An alternative way to sense infection is to detect danger signals such as cell
49 damage. Here, damaged cells release damage-associated molecular patterns
50 (DAMPs), which bind host receptors and trigger the immune response [2].
51 Finally, pathogens may be detected because of 'missing self'—they lack some
52 factor found on host cells that inhibits immune activation [3].
53
54 Sometimes, immune responses must be mounted against parasites that are
55 closely related to the host. For example, plants can be infected by other plants,
56 insects by other insects, and some mammals are even infected by transmissible
57 cancer cells derived from their own species [4]. In one of the few cases that have
58 been characterized, tomato plants have evolved a PRR to recognize a PAMP
59 produced by the pathogenic plant *Cuscuta reflexa* [5,6]. However, it is unclear
60 whether this will be more broadly true. In many cases, it may be difficult to
61 recognize PAMPs as there will be fewer conserved differences between closely
62 related pairs of hosts and pathogens. This problem may be exacerbated as there
63 is selection on the pathogen to escape recognition by losing their PAMPs, and
64 this may be easier to evolve if you are already similar to your host. If this is the
65 case, recognition may rely on danger signals or detecting missing-self.
66
67 This problem is especially acute for insects, as their most important parasites are
68 frequently other insects [7]. Many parasitoid wasps infect their insect hosts by
69 injecting eggs into their haemocoel. Typically, infection by parasitoids leads to
70 activation of an immune response that involves the formation of a cellular
71 capsule around the parasitoid egg that later becomes melanized [8–10]. The
72 cellular immune response to parasitoid wasps in *Drosophila melanogaster*
73 involves the differentiation of a hemocyte type rarely found in healthy larvae, the
74 lamellocyte [8]. These form the outer-layer of the cellular capsule around the
75 parasitoid egg, which is melanized when pro-phenoloxidase 2 and 3 (PPO2 and
76 PPO3) are activated in crystal cells and lamellocytes respectively [11].
77
78 The recognition of parasitoid infections relies in part on danger signals.
79 Lamellocyte differentiation can be triggered by sterile wounding of the larval
80 cuticle [12], which presumably mimics a parasitoid piercing the cuticle with its
81 ovipositor. Furthermore, in many insects, introducing inert objects into the
82 haemocoel leads to a cellular encapsulation response [13], and in species like *D.*
83 *yakuba*, this is accompanied by the object being melanized [14,15]. However, in
84 other cases these danger signals only lead to an incomplete immune response, as
85 a cellular capsule forms but is not melanized [16], or, in species like *D.*
86 *melanogaster,* there is only a low level of melanization [15,17]. Interestingly, *D.*
87 *melanogaster* larvae that have been parasitized by the wasp *Asobara tabida* are

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88 more likely to strongly melanize inert objects [17]. This suggests that a PAMP
89 injected by the parasitoid might activate the melanization response. Here, we
90 examine how the combination of danger signals and PAMPs activate different
91 components of the immune response of *D. melanogaster* to parasitoid wasps.
92
93
Results
94
95
Danger signals induce immune cell differentiation
96
97 Parasitoid wasp attack induces the rapid differentiation of blood cells called
98 lamellocytes, which encapsulate and melanize the wasp. It has previously been
99 reported that sterile wounding of larval cuticle induces lamellocyte
100 differentiation [12]. In line with this, we found that injecting a droplet of paraffin
101 oil induced lamellocyte differentiation (Figure 1A; main effect of treatment: F =
102 36.187, d.f = 2, 38, $p = 1.59 \times 10^{-9}$; oil vs. control t = 5.298, d.f. = 38, $p < 1.57 \times 10^{-5}$).
103
104 To examine the role of PAMPs in this response, we homogenized adult male
105 wasps in the paraffin oil before injecting the fly larvae. Therefore, this treatment
106 combines both the wounding from injection and exposure to PAMPs. We found
107 that the addition of wasp homogenate led to a larger number of lamellocytes
108 being produced (Figure 1A; oil vs. wasp homogenate t = 3.26, d.f. = 38, p=0.007).
109 This suggests that danger signals resulting from the wound created by the female
110 ovipositor are the primary factor triggering lamellocyte differentiation, but the
111 response is amplified by recognition of non-self from wasps.
112

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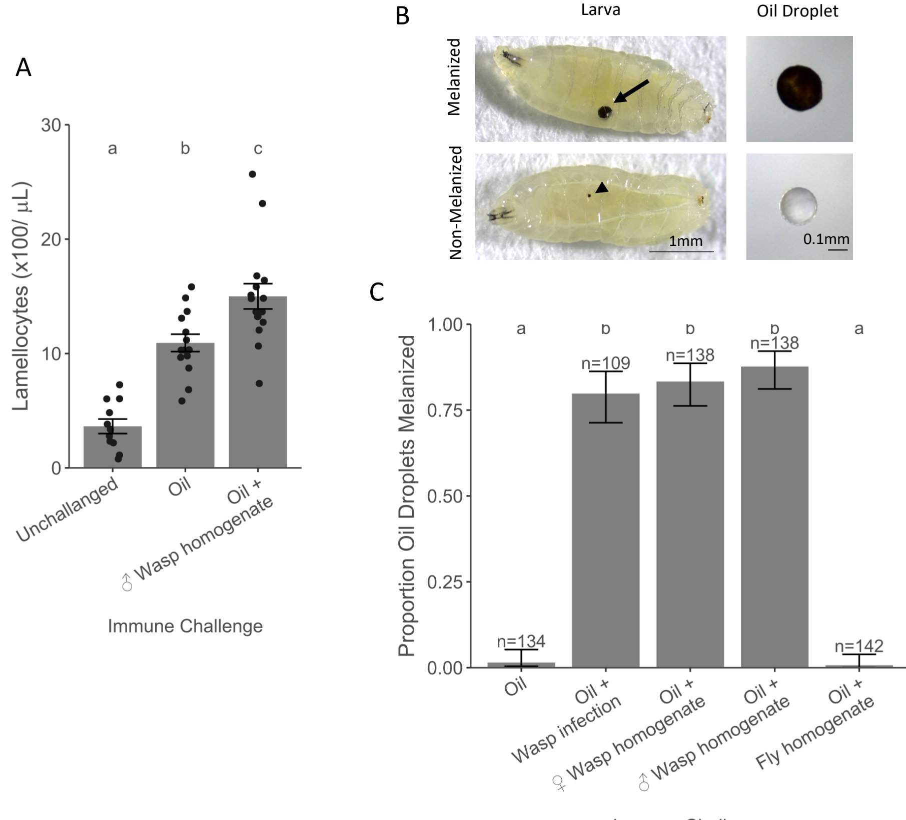

113
114 **Figure 1. The effect of parasitoid wasp exposure on the melanization of oil**
115 **droplets** (A) Concentration of lamellocytes in the hemolymph of unchallenged
116 larvae and larvae 48h post injection with oil or oil + wasp homogenate. The data
117 points are independent measurements on hemolymph pooled from 8-10 larvae. (B)
118 Oil droplets injected into larvae are either melanized (arrow) or not. Melanization of
119 cuticle resulting from injection wounding is often visible (arrow head). (C)
120 Proportion of larvae with melanized oil droplets 48h after different immune
121 challenges. Different letters represent treatments with statistically significant
122 differences (Tukey's Honest Significant Difference Test, $p<0.01$).
123

124 Parasite molecules activate the melanization response

125
126 The final step of the immune response against parasitoid wasps is the
127 melanization of the wasp egg. To test if this requires the recognition of non-self,
128 we examined whether paraffin oil droplets were melanized 48h after injection
129 (Figure 1B). Wounding alone was not sufficient to activate this response, as
130 paraffin oil by itself did not induce a strong melanization reaction (Figure1C).
131 However, if larvae were previously infected by a low virulence *L. boulardi* strain
132 (G486), the melanization of the oil droplet increases (Figure1C; main effect of
133 treatment: $X^2 = 577.39$, d.f = 4, $p < 2 \times 10^{-16}$; oil vs. oil + wasp infection: z = 7.612,
134 $p = 2 \times 69^{-13}$). Therefore, the presence of the parasite is required to trigger this
135 immune response.
136
137 To test whether parasitoid wasp PAMPs are responsible for the activation of the
138 immune response, we injected flies with paraffin oil containing homogenized
139 female wasps. This led to a robust melanization response that was

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140 indistinguishable from that seen when the flies had been parasitized (Figure 1C;
141 female wasp homogenate vs oil: z=7.967, p = 1 x 55^{-14}). Furthermore, this
142 reaction is not due to the presence of eggs or venoms in the female wasp
143 homogenates, as male wasp homogenates induced a similar response (Figure 1C;
144 male homogenate vs female homogenate: z=1.051, p=1).
145
146 The immune response to our crude homogenate of parasitoid wasp could be a
147 specific response to PAMPs in the parasitoid tissue or a general response to
148 injecting damaged cells, which are known to release DAMPs that activate the
149 innate immune system [18]. To distinguish these hypotheses, we injected larvae
150 with paraffin oil containing *D. melanogaster* homogenate. This did not induce
151 melanization (Figure 1C; oil vs fly homogenate: z=-0.634, p=1). Together, these
152 results indicate that the *D. melanogaster* immune system recognizes non-self
153 molecules in the parasitoid wasp to activate the melanization response.
154
155 To determine if the fly immune system recognizes wasp proteins, we treated the
156 wasp homogenate with proteinase K, a broad range serine protease [19]. This
157 did not reduce the proportion of melanized oil droplets (Figure S1, main effect of
158 treatment: X^2 = 102.24, d.f = 4, p < 2 x 10^{-16}; No autoclave vs. No autoclave +
159 Proteinase K: z = 1.267, p =1). Serendipitously, we tested samples where wasps
160 were autoclaved before homogenization. In itself this has no effect on
161 melanization rates (Autoclave vs. No autoclave: z = 1.254, p =1). However, when
162 the autoclaved homogenate is treated with proteinase K, the number of
163 melanized oil droplets is significantly reduced (Autoclave vs. Autoclave +
164 Proteinase K: z = 5.41, p =6.3 x 10^{-7}). This result suggests that one or more
165 proteins in the wasp body act as PAMPs to activate the fly immune system.
166
167
168 **PAMPs activate the humoral immune response**
169
170
171 In addition to the cellular immune response, the melanization of the capsule
172 formed around wasp eggs relies on a humoral immune response involving the
173 secretion of molecules from the fat body [20]. To understand the effects of
174 danger signals and wasp PAMPs on this response, we sequenced RNA extracted
175 from fat body 24h post injection of paraffin oil, paraffin oil with wasp
176 homogenate and from non-injured controls (unchallenged). After aligning the
177 RNA-seq reads to the *D. melanogaster* genome, the number of uniquely mapped
178 exonic reads ranged from 3,288,236 to 15,962,793 (Table 1B).
179
180 The injection of paraffin oil alone did not lead to the significant upregulation of
181 any genes at 24h, but the addition of wasp homogenate upregulated 29 genes
182 (Figure 2A-B). Ten of these encode serine proteases with a trypsin domain
183 (Table S2), a class of proteins known to be involved in the melanization cascade
184 and Toll pathway [20]. Other upregulated genes encode immunity-related
185 molecules, including Toll, thioester-containing proteins (TEPs) and a fibrinogen.
186
187 To confirm the specific response to wasp homogenate, we chose a subset of the
188 genes and analysed gene expression with qPCR. This revealed that wounding and

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189 the wasp PAMP elicit distinct humoral immune responses. The injection of
190 paraffin oil alone was sufficient to upregulate Bomanin genes, which encode
191 short peptides that play a role in killing bacterial pathogens [21] (Figure 2C;
192 Unchallenged vs Oil: p=0.04, p=0.05 and p=0.10). However, other genes
193 including secreted serine proteases, a lectin and a Tep were specifically
194 upregulated in the presence of the wasp homogenate (Figure 2C). This suggests
195 that a humoral immune response against bacteria can to triggered by wounding,
196 but PAMPs are required for the specific anti-parasitoid response.
197
198 Genes upregulated upon wasp homogenate injection include *Tep1* and *Tep2*
199 (Figure 2B-C), which encode secreted complement-like proteins. TEPs are
200 involved in resistance against bacteria, fungi and parasitoid wasps [22,23], and
201 in the mosquito *Anopheles gambiae* they act as an opsinin, binding to the
202 ookinetes of *Plasmodium* eggs [24]. We therefore tested the role of *Tep1* in the
203 melanization process. Knocking down *Tep1* expression with RNAi reduced the
204 ability of larvae to melanize the oil droplet prepared with wasp homogenate
205 (Figure 2D, main effect of treatment: X^2 = 16.367, d.f = 1, p = 4.525 x 10^{-5}).
206 Therefore, the wasp PAMPs upregulate genes required for the immune response
207 that kills the parasite.
208

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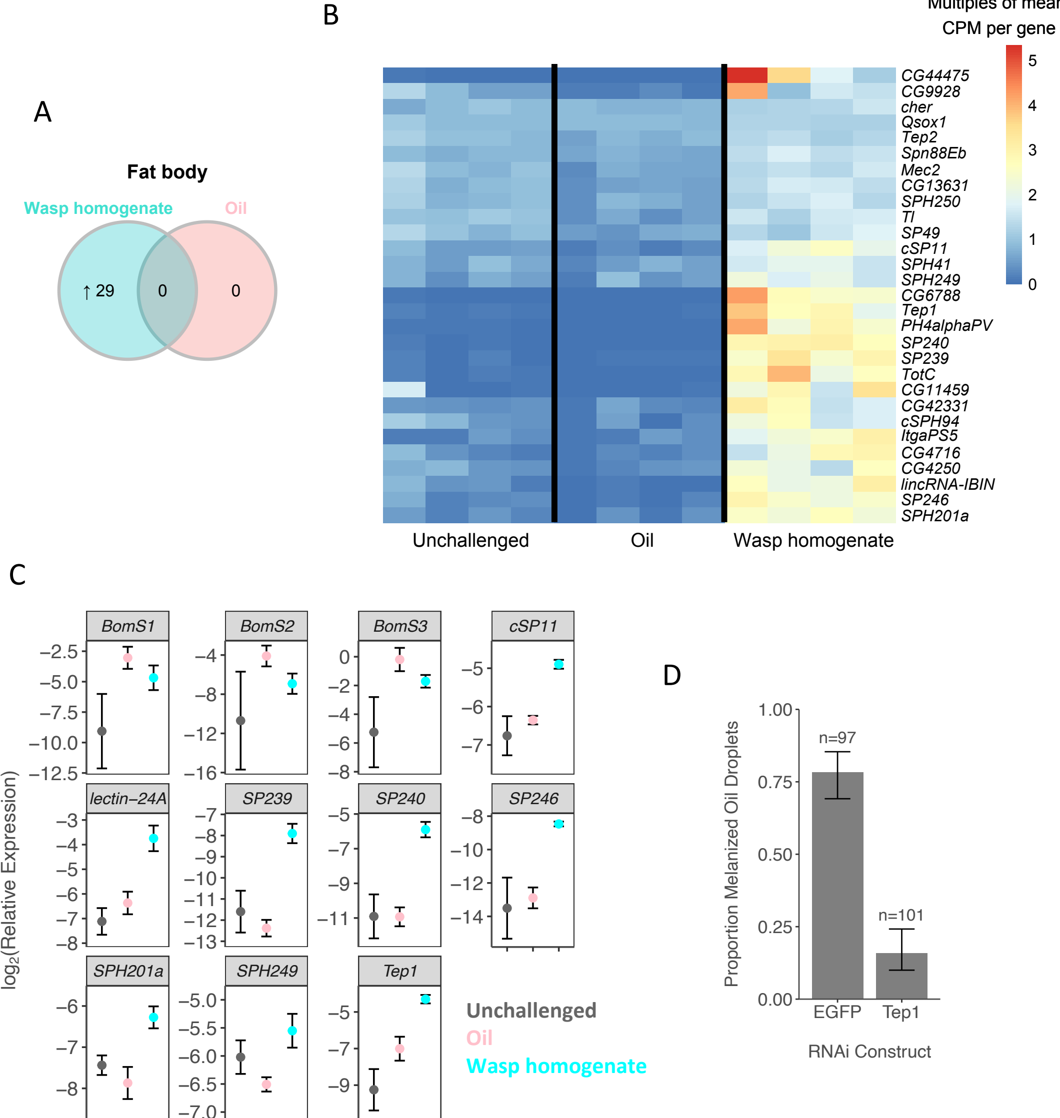

210
211
212
213 **Figure 2 – Transcriptional response of fat body to wasp exposure** Larvae were
214 injected with oil, wasp + oil or unchallenged. RNA from the fat body was sequenced
215 24h post treatment. (A) The number of genes with significantly changes in
216 expression compared with unchallenged conditions. (B) The expression of 29 genes
217 with significantly changes in expression after immune challenge. (C) The rate of
218 melanization of wasp + oil droplets in transgenic flies that express constructs to
219 knock down the expression of *Tep1* or eGFP (control).
220
221

222 Parasitoid wasp PAMPs amplify the transcriptional response of immune
223 cells to danger signals

224
225 To understand the role of PAMPs in the cellular immune response we repeated
226 the RNA-seq experiment on hemocytes. The number of uniquely mapped exonic
227 reads ranged from 2,520,358 to 22,036,188 (Table 1B). There is a much broader

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228 transcriptional change in hemocytes than in the fat body (Figure 3A), with 3,887
229 genes being differentially expressed after larvae were injected with wasp
230 homogenate (Figure 3A, Figure S2). The genes that were significantly
231 differentially expressed when larvae were injected with mineral oil alone were
232 largely a subset of these genes (Figure 3A). The wasp homogenate and mineral
233 oil injections are largely causing the same genes to change in expression, but the
234 magnitude of the transcriptional response is greater in the presence of the PAMP
235 (Figure 3B). Therefore, the PAMP amplifies the response to a danger signal.
236
237 The massive transcriptional response of hemocytes may reflect the
238 differentiation of lamellocytes, which are rare in homeostasis but increase after
239 wasp infection. Genes upregulated by wasp homogenate were enriched for
240 biological adhesion and cytoskeleton organization, which may be related to the
241 role of lamellocytes in capsule formation and the changes in cell morphology that
242 occur as these cells differentiate (Figure S3A). The upregulated genes were also
243 enriched for endocytosis, macroautophagy and other immune functions (Table
244 S3) [25]. In contrast, genes downregulated by wasp homogenate were enriched
245 for extracellular structure organization, a housekeeping function of
246 plasmatocytes (Figure S3A).

247 To test whether these transcriptional changes were linked to the differentiation
248 of lamellocytes, we compared our data to previous results we have generated
249 using single-cell RNA sequencing (scRNA-seq) [26]. We found that genes that
250 were highly expressed in lamellocytes were upregulated by injecting wasp
251 homogenate and vice-versa for downregulated genes (Figure S3B). To
252 investigate this further, we estimated the abundance of different hemocyte types
253 using digital cytometry [27]. This is a statistical technique that estimate cell
254 proportions in 'bulk' RNA-seq data using the single cell expression profile [26] as
255 a reference. We estimated that there was a moderate increase in the proportion
256 of lamellocytes following the injection of an oil droplet (Figure 3C). However, the
257 injection of wasp homogenate led to the differentiation of mature lamellocytes
258 (LAM3) together with large numbers of immature lamellocytes (Figure 3C).

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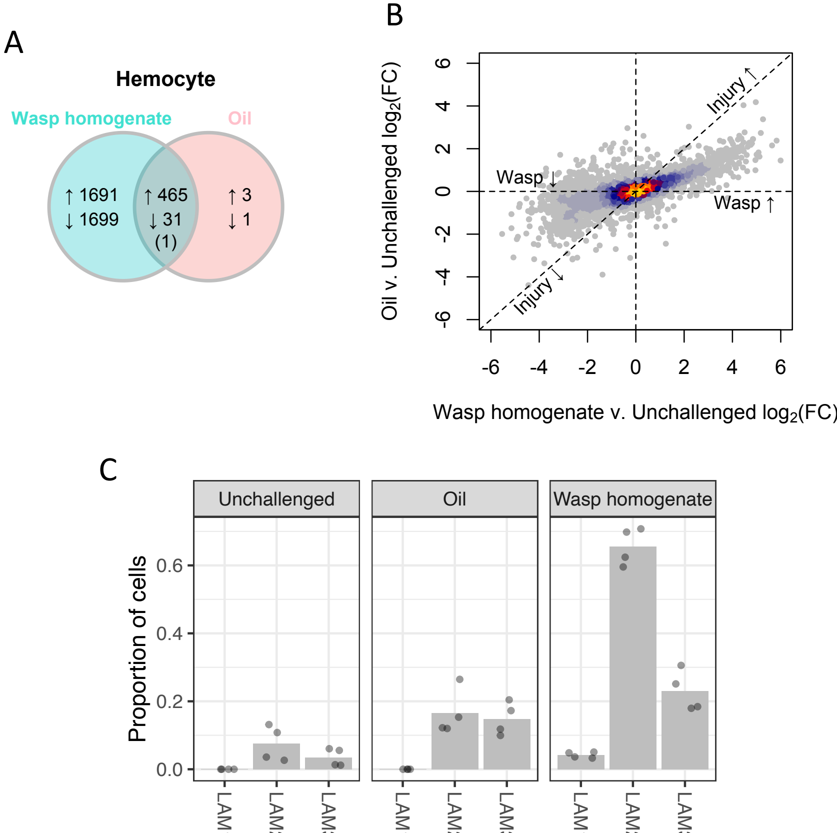

259
260 **Figure 3. Transcriptional response of hemocytes to wasp exposure** Larvae were
261 injected with oil, wasp + oil or unchallenged. RNA from the hemocytes was
262 sequenced 24h post treatment. (A) The number of genes with significantly changes
263 in expression compared with unchallenged conditions. (B) Changes in gene
264 expression induced by injection of wasp homogenate (x-axis) and by injection of oil (y-
265 axis). Because both treatments cause injury, genes solely regulated by injury will be
266 close to the dashed diagonal 1:1 line. Genes specifically activated by wasp PAMPs will be
267 on the x-axis. Relative expression is represented as $\log_2$(Fold Change). (C) Inferred
268 proportion of immature (LAM1 and LAM2) and mature (LAM3) lamellocytes estimated
269 from the RNA-seq data using digital cytometry. Each point is an independent sample and
270 the bars are the mean.
271
272
273 ## *Drosophila melanogaster* has evolved to recognize parasitoid-specific
274 molecules
275
276 To test if *Drosophila* larvae have evolved to recognize parasitoid-specific
277 molecules, we injected fly larvae with homogenates from prepared from 44
278 insect species and examined whether they activated the melanization response
279 to oil droplets. The two parasitoid species resulted in the two highest
280 melanization rates of all 44 species, and this parasitoid-specific response was
281 significant after correcting for the phylogenetic relatedness of the 44 species
282 (Figure 4; phylogenetic mixed model: p=0.006). Therefore, the *Drosophila*

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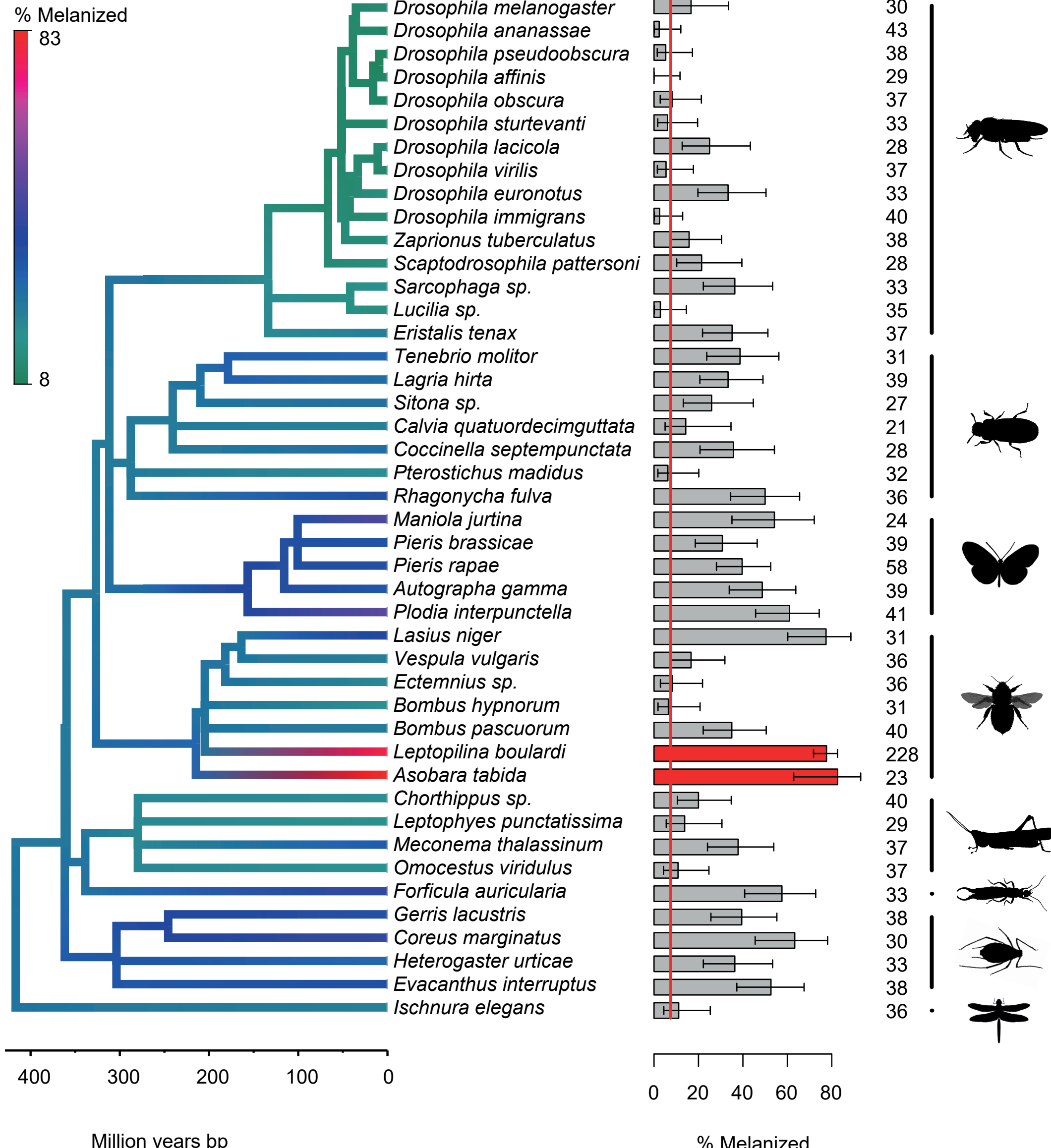
283 immune system appears to have evolved a specific mechanism to recognize
284 parasitoids.
285
286 Despite this parasitoid-specific response, homogenates of many other insect
287 species cause some level of immune activation (Figure 4). It is striking that
288 species closely related to *D. melanogaster* do not activate the melanization
289 response (Figure 4; 95% confidence intervals overlap the dashed line basal
290 response). Therefore, it appears that many insect species contain factors that
291 cause some activation of the melanization response, but *Drosophila* immunity is
292 not activated by self.



293
294
295 **Figure 4. The effect of injecting homogenates of different insects on the**
296 **melanization of oil droplets.** The bar chart shows the proportion of oil droplets
297 that were melanized with 95% binomial confidence intervals. The red line is the rate
298 at which oil droplets were melanized without any insect homogenate (*N*=241). The
299 red bars are parasitoids of *D. melanogaster.* Sample sizes are given beside the bars.
300 The silhouettes represent different insect orders (from top: Diptera, Coleoptera,
301 Lepidoptera, Hymenoptera, Orthoptera, Dermaptera, Hemiptera, Odonata).

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Discussion

Immune responses are multistep processes that require several levels of regulation. Here, we describe how the immune response of *D. melanogaster* against parasitoid wasps is regulated by two modes of immune activation. In line with previous results [12], immune challenge with an inert object induces the differentiation of lamellocytes. The signal required for this is likely a damage associated molecular pattern (DAMP) produced during wounding [28]. However, the final step of the encapsulation response—melanization—only occurs when wasp molecules are present. This suggests that this part of the encapsulation response is activated by a pathogen associated molecular pattern (PAMP). This PAMP has different effects on the two main immune tissues of *Drosophila*. In hemocytes, it enhances the differentiation of lamellocytes, amplifying the effects wounding. In the fat body it triggers the upregulation of a small number of humoral immune genes whose expression is not affected by wounding alone. This includes the secreted complement-like molecule TEP1, which we show is required for the melanization response.

The reason why this immune response relies on both PAMPs and DAMPs is unknown. However, the PAMP may be used to adjust the immune response to target this specific parasite, as wounding may occur for many other reasons. Alternatively, it may allow the response to be modulated according to the levels of infection (although this may be less relevant to parasitoids than other forms of infection). This regulation step may be important for the fly to minimize costs associated with the activation of the melanization cascade. Artificial activation of the melanization response is detrimental for host tissue physiology [29], while the production of lamellocytes, that is enhanced by the PAMP, is thought to be energetically costly for the fly [30]. Without this step of immune regulation, any cuticular wound would result in activation of the melanization cascade and incur associated costs.

Homogenates prepared with other insect species can also result in melanization, but parasitoid wasps induce the highest response. This variation in the phenotype may be the result of recognition of different molecules or different quantities of the same molecule in different species. It is worth noticing that homogenates prepared with other *Drosophila* species induce a very poor response. This is in contrast with a previous report that shows encapsulation and melanization of fat bodies from heterospecific tissue transplants when the donor is a *Drosophila* species outside the melanogaster group [31]. In this case, host larvae are mutants in an unidentified gene that causes the differentiation of lamellocytes in homeostasis, possibly explaining the discrepancy with the results presented here.

All major macromolecules, proteins, carbohydrates, nucleic acids, and lipids can act as PAMPs [1], and the crude wasp homogenate used in our experiments includes all these molecules. However, we found that treatment with proteinase K reduces the activity of the PAMP, suggesting that the active molecule may be a

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351 protein or glycoprotein. Unexpectedly, the proteinase K was only effective if the
352 wasps were autoclaved prior to homogenization. This suggests that the wasp
353 protein may be resistant to proteinase K digestion. Proteinase K resistant
354 proteins are rare [32] but they include some eukaryotic proteins [33]. The high
355 temperature treatment may have modified the configuration of the wasp PAMP
356 and made it accessible to proteinase K digestion.
357
358 In conclusion, we show that *Drosophila*'s immune system can recognize the
359 presence of wasp parasites and uses this recognition to modulate the cellular
360 and humoral responses that are initiated by injury. To our knowledge, this is the
361 first report of an animal immune system recognizing a PAMP from a parasite that
362 is closely related to itself.
363
364 **Material and methods**
365
366 *D. melanogaster* and *L. boulardi* maintenance
367 For all experiments except RNAi we used an outbred *D. melanogaster* population
368 that was established from 372 isofemales caught in Cambridgeshire in October
369 2017. Population size was maintained over 500 flies per generation and had over
370 10 generations of laboratory adaptation before the start of experiments. For
371 experimental procedures, flies were allowed to lay eggs overnight on agar plates
372 covered with yeast (*Saccharomyces cerevisiae* – Sigma YSC2). Eggs were washed
373 from the agar plate with PBS and transferred into 1.5ml microcentrifuge tubes.
374 13μl of eggs and PBS (~150 eggs) were transferred onto 50mm cornmeal food
375 plates. These were incubated for 72 hours before experiments. Developing and
376 adult *D. melanogaster* were maintained at 25°C, 70% relative humidity in an 8h-
377 16h dark-light cycle.
378
379 *L. boulardi* was maintained by allowing females to infect 1st instar larvae of the
380 outbred population and incubating them at 25°C. Adult wasps were collected 24
381 days after infection and maintained at room temperature with a drop of honey
382 for up a maximum of 5 days before infections. To infect *D. melanogaster* larvae, 3
383 *L. boulardi* females were allowed to infect larvae on the cornmeal food plates for
384 3 hours.
385
386 RNAi
387 The following *D.melanogaster* stocks were obtained from the Bloomington
388 Drosophila Stock Center: UAS- Tep1^{dsRNA} (BL#: 32856), UAS-EGFP^{dsRNA} (BL#:
389 41556) and dautherless-GAL4 (da-GAL4, BL#: 27608). Balancer chromosomes
390 from stock 27608 were substituted with autosomes from stock w^{1118}. Females
391 from the ubiquitously expressed GAL4 driver, da-GAL4, were crossed with males
392 from both UAS-dsRNA lines. Larvae from this cross were injected and analyzed
393 as described below.
394
395 Insect species
396 We used 44 species of insects to test if they activated the melanization response.
397 Drosophilid species (kind gift from Ben Longdon), *A. tabida* strain SFA3
398 (collected in Sainte Foy-Lès-Lyon, Rhône, France in 2012 and provided by
399 Fabrice Vavre) and *L. boulardi* strain G486 [34] were lab maintained stocks. All

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400 other species were collected in Cambridge, UK, in July 2018 and identified
401 morphologically. For large species a single specimen was collected, while for
402 smaller species multiple individuals were pooled.
403
404 Oil Injections
405 To test whether insect extracts could activate the immune response, we
406 homogenized insects in paraffin oil. Our initial characterization of wasp extracts
407 used 20 female *L. boulardi* in 200µl of paraffin oil (Sigma-Aldrich M5904;
408 approximately 0.025mg wasp/µl oil). In the experiment involving multiple
409 species, specimens were weighed and paraffin oil was added to reach a
410 concentration of 0.025mg/µl. For large specimens the thorax was used, while for
411 small specimens the entire animal was used (body part did not have a significant
412 effect on melanization rates). Specimens were homogenized in the paraffin oil
413 with a pestle in 0.5ml microcentrifuge tubes. To remove large particles, the
414 solution was centrifuged for 1min at 300g and the supernatant was transferred
415 to a new 0.5ml microcentrifuge tube.
416
417 Borosilicate glass 3.5" capillaries (Drummond Scientific Co. 3-000-203-G/X)
418 were pulled to form thin needles in a needle puller (Narishige PC-10). The needle
419 was backfilled with the oil solution with a syringe and attached to a nanoinjector
420 (Drummond Scientific Co. Nonoject II). Late 2nd instar and early 3rd instar larvae
421 were carefully removed with forceps from cornmeal food plates and placed on
422 filter paper, in groups of 10. Larvae were carefully injected with 4.6nl of solution.
423 After injection, ddH20 was added with a brush to remove the larvae and 40
424 larvae were transferred into a cornmeal food vial at 25°C, 70% relative humidity
425 and an 8:16 dark:light cycle. After 48 hours larvae were removed with a 15%
426 w/v sugar solution and scored for total melanization of the oil droplet.
427
428 Hemocyte counts
429 To count hemocytes, larvae were injected as described above. After 48 hours,
430 injected and control larvae were collected, washed in PBS, dried on filter paper
431 and pooled in groups of 8 to 10 larvae in a well of a multi-well porcelain plate.
432 Larvae were rapidly dissected with a pair of forceps from the ventral side.
433 Hemolymph was recovered with a 1-10µl micropipette and transferred into a
434 0.5ml microcentrifuge tube. 1µl of hemolymph was collected, diluted in 9ul of
435 Neutral Red solution (1.65g/L PBS – Sigma-Aldrich N2889) and thoroughly
436 mixed. The hemolymph dilution was transferred into a counting Thoma chamber
437 (Marienfeld #0640711) and hemocytes were counted in a total volume of 0.1µl
438 with a 40x objective (Leica DM750). Lamellocytes were distinguished from
439 plasmatocytes and crystal cells by morphology.
440
441 RNA sequencing
442 We performed RNA-seq on flies injected with wasp homogenate or oil droplets
443 and unchallenged flies. Hemocytes from ~100 larvae were pooled in 100µl of
444 PBS, 24 hours after injections. Fat body samples were dissected from 8 third
445 instar larvae and pooled in 100ul of PBS. RNA was purified from hemolymph or
446 fat body samples in an identical manner: 1ml TRIzol [Ambion: 15596018] was
447 added to collected tissue and the samples were homogenized by pipetting
448 several times. 200ul of chloroform [Fisher Scientific: C/4920/08] was added;

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449 samples were shaken for 15 seconds, incubated at room temperature for 3
450 minutes then centrifuged at 12,000g for 10 minutes at 4°C. The upper aqueous
451 phase (approximately 500ul) was removed to a fresh tube and RNA was
452 precipitated by adding 2.5 volumes of isopropanol and incubated at -20°C for 1
453 hour. RNA was pelleted by centrifugation, washed with 70% ethanol, and re-
454 suspended in 15ul of nuclease free water [Ambion: AM9930]. RNA was
455 quantified by Qubit fluorometer2.0 [ThermoFisher Scientific: Q32866] with the
456 Qubit RNA HS Assay Kit [ThermoFisher Scientific: Q32852] and integrity was
457 assessed by gel electrophoresis. 100-4,000ng of RNA was used for RNA-Seq
458 library preparation.
459
460 Libraries were prepared using the KAPA Stranded mRNA-Seq Kit Illumina®
461 platform. TrueSeq DNA Low Throughput adaptors used were from Illumina®
462 TruSeq™ KAPA Si adaptor kit KK8701 and adaptor concentrations and the
463 number of PCR cycles used to amplify the final libraries were adjusted to the
464 total amount of RNA used for each library. Seven hemocyte libraries that gave a
465 low final concentration (<2ng/ul) were re-amplified for four more cycles. Quality
466 control of the libraries to ensure no adapter dimers were present was carried
467 out by examining 1ul of a 1:5 dilution on a High Sensitivity DNA chip (Agilent
468 Technologies: 5067-4626) on an Agilent 2100 Bioanalyzer. The average library
469 size including adapters was 350bp. Sequencing was carried out at the Cancer
470 Research UK Cambridge Institute in June 2019. All 24 libraries were multiplexed
471 and sequenced on one lane of HiSeq4000 using 50bp single end reads.
472
473 Differential expression tests
474 Sequenced RNA-seq reads were trimmed and aligned to the *D. melanogaster*
475 genome and reads counts per gene were estimated. Using Trimmomatic v.0.36
476 [35], we clipped adaptors sequences, removed the first three and last three
477 bases, filtered strings of low-quality bases found in 4bp sliding windows where
478 quality dropped below 20 and ensured that the remaining reads had a minimum
479 size of 36bp. We mapped the resulting reads using STAR v2.6 [36] to the *D.*
480 *melanogaster* reference (r6.28) [37] attained from Flybase (FB2019_02) [38]. We
481 prepared the genome for STAR mapping using a sjdbOverhang of 49. Then, we
482 mapped reads using the basic option for the twopassMode parameter, filtered
483 multi-mapped reads and sorted the remainder by coordinates. We used
484 featureCounts [39] to compute read counts for genes using their Flybase IDs. We
485 only considered reads with a minimum quality score of 10.
486
487 We performed differential expression tests for the fat body and hemocyte
488 libraries separately using edgeR v.3.24.3 [40,41] and limma v.3.38.3 [42]. We
489 only kept genes that had CPM greater than or equal to 2 in at least four samples
490 for a given tissue. We normalized read counts using trimmed mean of M-values.
491 For a given tissue, we had four replicate libraries for each of three groups: wasp
492 homogenate, oil and unchallenged. Salivary gland and male germ tissues were
493 difficult to exclude completely when dissecting larvae and isolating the fat body
494 of *D. melanogaster*. To minimize noise in our differential expression tests
495 attributable to this limitation, we excluded genes that had enriched expression in

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496 the aforementioned tissues. We obtained tissue level RNA-seq expression data
497 from FlyAtlas2 [43] and calculate the tissue specificity index (Tau) [44] for each
498 gene in the larvae and adult males separately. We then excluded tissue-specific
499 fat body expressed genes (Tau >0.8) with greatest expression either in the larval
500 salivary gland or adult male testes. We only excluded genes that had FPKM >1 in
501 either of those two tissues in FlyAltas2. We fit a linear model using limma
502 contrasting gene expression among the three groups (full model in script). After
503 checking that the mean-variance trends followed the expected dispersion, we fit
504 contrasts and used the *eBayes* function to uncover genes with evidence of
505 significant differential expression between the wasp homogenate and
506 unchallenged comparison and the oil and unchallenged comparison separately.
507 We used the *heatscatter* function from the LSD v.4 R package to compare the
508 $\log_2$FC in expression between the two comparisons. We then extracted genes
509 with *P*-values of less than 0.05 after a false discovery rate (FDR) correction. For a
510 few of these genes, we divided counts per million reads (CPM) for each library by
511 the overall total across all libraries per gene to compare expression levels across
512 samples. We plotted a heatmap of relative gene expression using the pheatmap
513 v.1.0.12 R package. Serine proteases were named according to [45]. Log_2CPM
514 counts for all genes (unfiltered) in hemocyte and fat body tissues is accessible
515 from the following gene expression browser
516 https://arunkuma.shinyapps.io/waspapp/ (last accessed June 2022). We
517 performed gene ontology (Huang et al., 2009) enrichment analyses on the
518 differentially expressed genes using Flymine [46]. The genes detected in each
519 tissue was used as the background list. Non-redundant gene ontology terms
520 were identified using REVIGO [47] keeping FDR *P*-values < 0.05 and similarity =
521 0.4.
522
523 Bulk RNA-seq deconvolution
524 We used the digital cytometric method CIBERSORTx [27] to infer the proportion
525 of hemocyte clusters, which were identified in [26], in the bulk RNA-seq data. We
526 first created a signature matrix using read counts from 2,000 highly variable
527 genes in the scRNA-seq. Between 300 to 500 genes were used for barcoding cell
528 types and a q-value of 0.01 was used to test for the significance of differential
529 gene expression. Quantile normalization was disabled as recommended and a
530 maximum conditional number of 999 was used by default. Only genes with
531 average $\log_2$ expression of 0.5 were analysed. Five replicates were used to build
532 the scRNA-seq reference file. Half of available gene expression profiles were
533 randomly selected to generate the file. Then, we imputed cell fractions using the
534 bulk RNA-seq read counts from hemocyte libraries with an S-mode batch
535 correction and used 100 permutations to assess the significance of cluster
536 inferences.
537
538 Gene expression by qPCR
539 To analyze the expression by qPCR, RNA was extracted from pools of 10 larvae,
540 48 hours post injection. Larvae were homogenized in 250µl TRIzol [Ambion
541 15596018] with ~10 1.0mm zirconia beads [Thistle Scientific] in a tissuelyser
542 [Retsch MM300] and kept at -80°C. For RNA extraction, samples were defrosted
543 and centrifuged for 10min at 4°C at 12,000g. 160µl of supernatant was
544 transferred into 1.5ml microcentrifuge tubes, 62.5µl of chloroform [Fisher

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545 Scientific C/4920/08] was added, tubes were shaken for 15s and incubated for
546 3min. After a 10min centrifugation at 12,000g at 4°C, 66µl of the aqueous phase
547 was transferred into a 1.5µl microcentrifuge tube, 156µl of isopropanol
548 [Honeywell 33539] added and the solution thoroughly mixed. After 10min
549 incubation samples were centrifuged for 10min at 12,000g at 4°C and the
550 supernatant was removed. RNA was washed with 250µl 70% ethanol,
551 centrifuged for 2min at 12,000g at 4°C. Ethanol was removed, samples dried,
552 20µl of nuclease free water [Ambion AM9930] was added and samples incubated
553 at 45°C for 10min. cDNA was prepared from RNA samples with GoScript reverse
554 transcriptase (Promega) according to manufacturer instructions. cDNA was
555 diluted 1:10. Exonic primers for *D. melanogaster* immunity genes were designed
556 in NCBI Primer-BLAST online tool (Table S4). The gene *RpL32* was used to
557 normalize gene expression (RpL32_qPCR_F-d: 5'-TGCTAAGCTGTCGCACAAATGG-
558 3'; RpL_qPCR_R-h 5'- TGCGCTTGTTCGATCCGTAAC-3'; Longdon et al. 2011).
559 Sensifast Hi-Rox SyBr kit [Bioline, BIO-92005] was used to perform the RT-qPCR
560 on a StepOnePlus system [Applied Biosystems]. Each sample was duplicated
561 (qPCR technical replica). The PCR cycle was 95°C for 2min followed by 40 cycles
562 of 95°C for 5s, 60°C for 30s. For one experimental replicate, we averaged the
563 cycle threshold (*Ct*) values of 4 biological replicates (groups of 10 larvae). The
564 relative expression of the gene of interest (GOI) was calculated as $2^{-\Delta\Delta Ct}$, where
565 $\Delta\Delta Ct = (Ct_{GOI(Treatment)} - Ct_{RpL32(Treatment)}) - (Ct_{GOI(Control)} - Ct_{RpL32(Control)})$.
566
567 Statistical analysis
568 The effects of different treatments on oil droplet melanization were analyzed
569 with a quasibinomial generalized linear model, with the ratio of melanized to
570 non-melanized oil droplets as a response and treatment as a fixed effect. We
571 used Tukey's honest significant difference test to compare treatments. To test
572 differences in lamellocyte numbers with different treatments we used a one-way
573 ANOVA with Tukey's test to compare treatments. We compared gene expression
574 ($2^{-\Delta\Delta Ct}$) using a two-tailed *t*-test, correcting *P*-values with the Bonferroni method.
575
576 We used a phylogenetic mixed model to analyze the effect of extracts of 44 insect
577 species on oil droplet melanization. This allowed us both to reconstruct ancestral
578 states across a phylogeny, and test whether *Drosophila* has evolved to
579 specifically recognized parasitoid wasps after correcting for the confounding
580 effect of the insect phylogeny. The ratio of melanized to non-melanized oil
581 droplets was the binomial response variable. Whether or not the insect was a
582 parasitoid was treated as a fixed effect. The phylogeny was treated as a random
583 effect, which allows the correlation between two species to be inversely
584 proportional to the time since those species shared a common ancestor
585 (following a Brownian model of evolution). A residual variance allowed for
586 differences between species that are unrelated to the phylogeny. We used the
587 phylogeny of the 44 insect species available through TimeTree (Kumar et al.
588 2017). The model was fitted using a Bayesian approach using MCMCglmm
589 (Hadfield 2010) using an inverse gamma prior. We ran 10^6 burn-in iterations
590 followed 10^7 iterations, sampling every 10^4 iterations.
591
592 R v3.6/4 [49] and RStudio v1.2.5042 [50] were widely used for generating
593 figures.

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594
595 **Author's contributions**
596 F.J. and A.B.L. conceived the study. N.H., A.D., J.P.D. and A.B.L. collected data.
597 M.P.H collected and identified the insect species. R.A., A.B.L. and F.J. analyzed the
598 data. All authors contributed to interpret the data and write the manuscript.
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607
608 **Data availability**
609 Raw and processed data files used to generate figures and the lists of
610 differentially expressed genes are available in NERC EDS Environmental
611 Information Data Centre (https://doi.org/10.5285/06ea87f3-476d-40fd-acce-
612 e6923e786d48). Scripts to analyze data are available in the Github (DOI:
613 10.5281/zenodo.6684609). Paired end reads from oil and wasp injections were
614 deposited into the NCBI Sequence Read Archive (SRA) and can be accessed with
615 Bioproject ID PRJNA685781.
616
617
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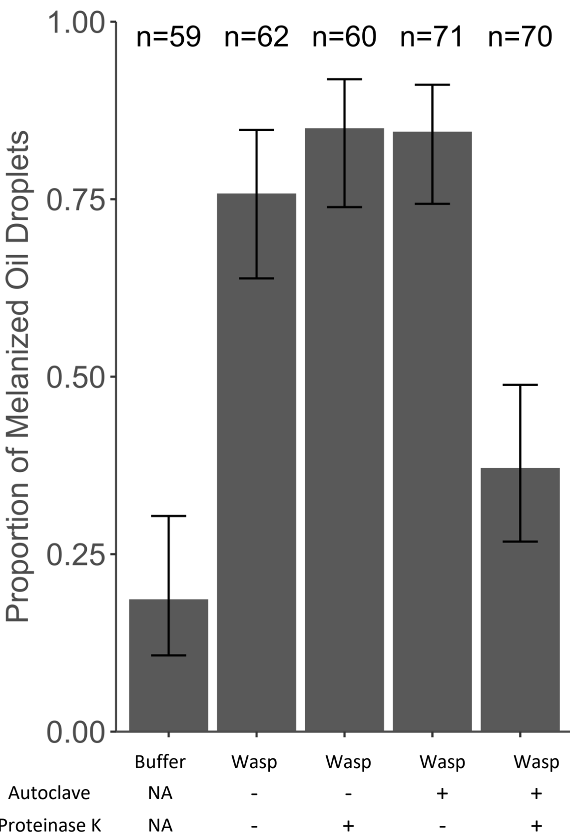

Figure S1 – Treatment of wasp homogenate with proteinase K. Larvae were injected with oil after a first injection with buffer or wasp homogenate. Wasps were autoclaved (+) or immediately frozen (-) before homogenization in buffer. Wasp homogenates were then treated with proteinase K (+) or incubated without proteinase K (-) before injection. Number of phenotyped larvae is shown and error bars represent standard error.

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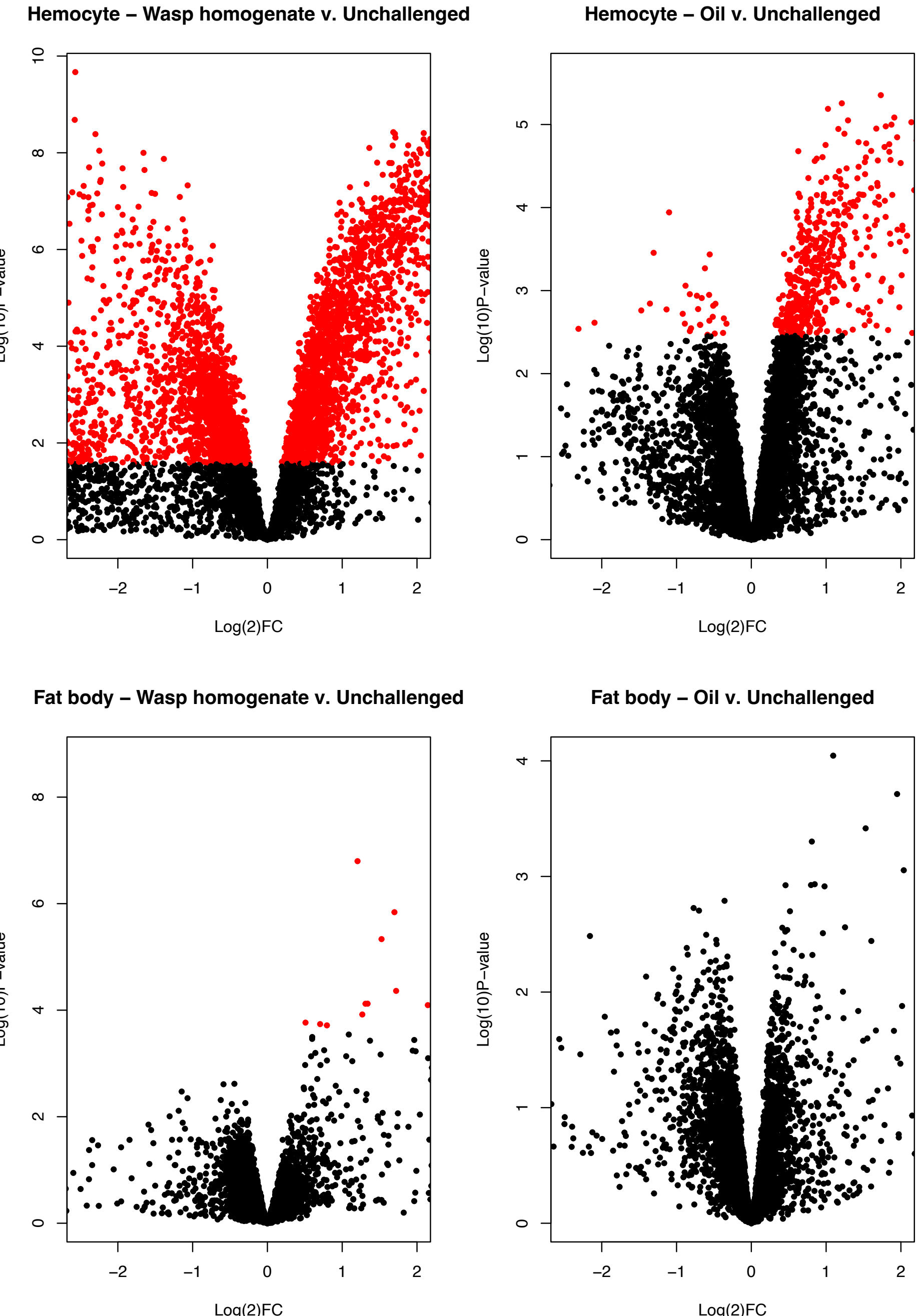

Figure S2. Volcano plots contrasting $\log_2$ fold change in gene expression against *P*-values generated from differential expression tests, for hemocyte and fat body samples. Red points indicate genes with false-discovery rate corrected *P*-values < 0.05.

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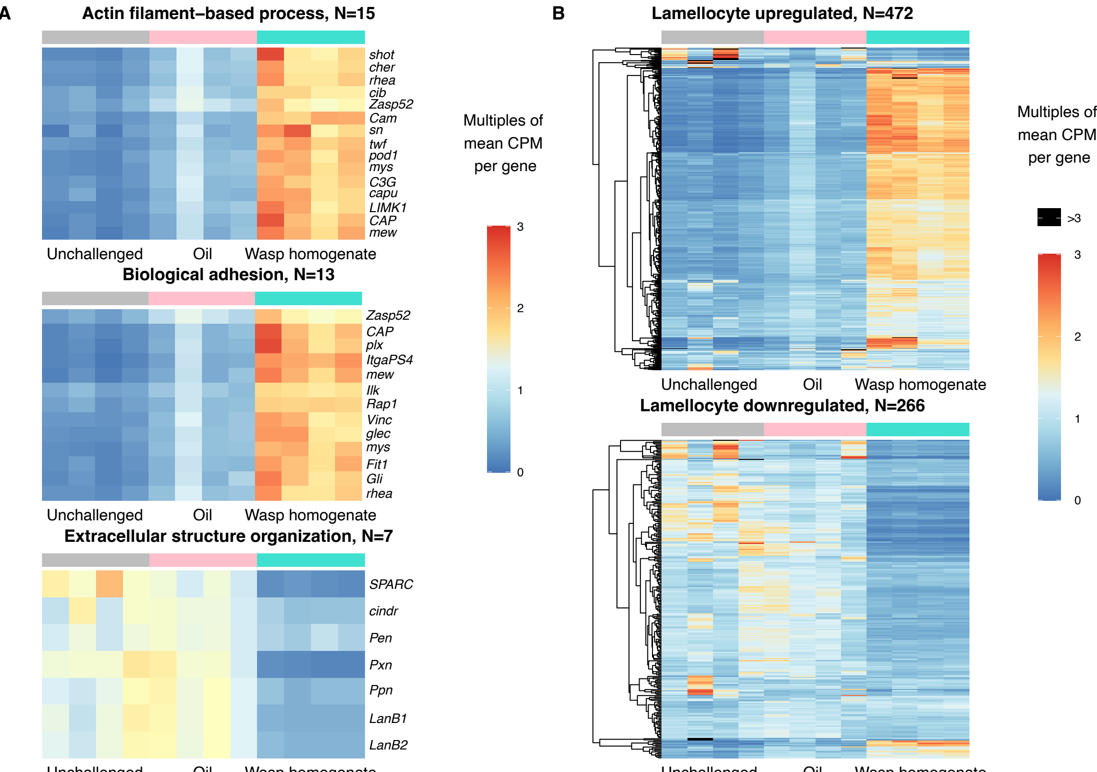

Figure S3. Heatmap of lamellocyte marker genes. Gene lists were attained from (Leitão et al. 2020). (**A**) Heatmap of genes part of gene ontology categories that are enriched in lamellocytes and plasmatocytes. (**B**) Heatmap of genes differentially expressed in mature lamellocytes compared to their plasmatocyte progenitors. The colour bar represents multiples of the mean counts per million (CPM) for each gene. As only few genes/samples had values >3, they are depicted as black.

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791 **Table S1.** Quality and read mapping metrics for the 12 hemocyte and 12 fat
792 body samples.
793
794 **Table S2.** Gene ontology (GO) terms, Kegg/Reactome pathways and Interpro
795 protein that are significantly enriched in the fat body genes upregulated in
796 wasp homogenate compared to unchallenged.
797
798 **Table S3.** Gene ontology (GO) terms, Kegg/Reactome pathways and Interpro
799 protein domains that are significantly enriched in the differentially expressed
800 genes in wasp homogenate compared to unchallenged in hemocytes.
801 Redundant GO categories were identified using REVIGO.
802
803 **Table S4.** List of primers used for qPCR.