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Does cold activate the Drosophila melanogaster immune system?

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Abstract

Cold exposure appears to activate aspects of the insect immune system; however, the functional significance of the relationship between cold and immunity is unclear. Insect success at low temperatures is shaped in part by interactions with biotic stressors, such as pathogens, thus it is important to understand how and why immunity might be activated by cold. Here we explore which components of the immune system are activated, and whether those components differ among different kinds of cold exposure. We exposed *Drosophila melanogaster* to both acute (2h, -2ºC) and sustained (10h, -0.5ºC) cold, and measured potential (antimicrobial peptide expression, phenoloxidase activity, haemocyte counts) and realised (survival of fungal infection, wound-induced melanisation, bacterial clearance) immunity following recovery. Acute cold increased circulating haemocyte concentration and the expression of *Turandot-A* and *diptericin,* but elicited a short-term decrease in the clearance of gram-positive bacteria. Sustained cold increased the expression of *Turandot-A*, with no effect on other measures of potential or realised immunity. We show that measures of potential immunity were up-regulated by cold, whereas realised immunity was either unaffected or down-regulated. Thus, we hypothesize that cold-activation of potential immunity in *Drosophila* may be a compensatory mechanism to maintain stable immune function during or after low temperature exposure.

Key Words: thermal biology, immune system, trade-off, cross-talk, damage

1. Introduction

Understanding how the physiological responses by animals to multiple biotic and abiotic stressors are linked through cross-talk or cross-tolerance is key to understanding the multidimensional impacts of a changing climate (Kaunisto et al., 2016; Sinclair et al., 2013). For example, insects appear to increase immune activity after cold exposure (Marshall and Sinclair, 2011; Zhang et al., 2011), which could modify host-pathogen relationships with changing winters (Williams et al., 2015). However, it is unclear whether these putatively-linked responses to distinct stressors are non-adaptive by-products of a generalised stress response, or the result of a functional coadaptation reflecting a link between pathogens and cold (Sinclair et al., 2013).

There are several, non-mutually exclusive hypotheses to explain why cold exposure might increase immune activity in insects. Cold exposure may non-adaptively activate immunity through activation of pathways shared with the stress response (Sinclair et al., 2013); however, recovery from cold exposure is already energetically costly (MacMillan et al., 2012), and it should be selectively disadvantageous to increase the cost associated with cold exposure by unnecessarily increasing resistance to pathogens. Instead, cold-induced immune activity may be an adaptive response to immune stress associated with cold (Sinclair et al., 2013). Among adaptive responses, immune processes could be required for repair of damage from cold exposure, or the presence of cold-active pathogens may have selected for increased immune activity following exposure to low temperatures (Sinclair et al., 2013).

To begin to understand the functional significance of cold-induced immunity, we can explore the effects of cold exposure on multiple components of the immune system. Measures of

53 potential immunity (*sensu* Fedorka et al., 2007), such as gene expression, provide insight into
54 affected pathways (e.g. Toll vs. IMD), and the potential for shared responses between the
55 immune system and the stress response. Measures of realised immunity (*sensu* Fedorka et al.,
56 2007) provide insight into whether or not the ability to fight or survive pathogen infection is
57 affected by cold exposure, and thus whether immune activation may be a response to pathogen
58 threat at low temperatures.
59
60 The physiological responses to cold differ among insects and kinds of cold exposure
61 (Zachariassen, 1985). Specifically, brief exposure to intense cold (e.g. 2 h at -5 °C in *D.*
62 *melanogaster*) is thought to cause cold shock injury, such as membrane phase transition (Drobnis
63 et al., 1993) or initiation of apoptosis (Yi et al., 2007), while longer cold exposures appear to
64 cause stress by disrupting ion and water balance (MacMillan et al., 2012). We can use these
65 differences in the physiological response to different types of cold exposure to further explore
66 the function of cold-induced up-regulation of immunity. For example, in *Drosophila*
67 *melanogaster,* short, prolonged, and repeated cold exposures elicit unique transcriptomic profiles
68 including of the expression of genes associated with immunity (Zhang et al., 2011), which
69 suggests that the relationship between cold and immunity depends on the physiological response
70 to a particular type of cold exposure. Therefore, if a suite of immune responses is specific to a
71 particular type of cold exposure, we can begin to infer how this activity is linked to the
72 physiological response to cold, and thus why immune activity might be activated.
73
74 To begin to understand why the immune system is linked to cold exposure, and what the
75 consequences of this relationship may be, we quantified multiple components of both potential

and realised immunity (Fedorka et al., 2007) in *Drosophila melanogaster* following two cold exposures that differed in both duration and temperature, and which are likely to provoke different physiological responses to cold, and which have previously been shown to elicit upregulation of immune-related genes (Zhang et al., 2011): acute (2h at -2°C) and sustained (10h at -0.5°C). Overall, we aim to use cold-activation of insect immunity as a system in which to tease apart the links between responses to abiotic and biotic stressors, and highlight the importance of considering the physiological connections between different stressors in insects.

2. Materials and methods

2.1 Rearing and cold exposures

An outbred mass-reared population of wild-type *Drosophila melanogaster* collected in London and Niagara-on-the-Lake, ON, Canada, in 2008 (described by (Marshall and Sinclair, 2010) was reared on a banana-cornmeal-agar medium at 21.5°C, 60% RH, under 14L:10D. Before beginning the experiment, we reared flies for several generations on a medium containing tetracycline and methylparaben (to eliminate *Wolbachia* sp., confirmed with PCR) (Carrington et al., 2010); flies used in experiments were then reared for at least four generations on banana-cornmeal-agar medium without antibiotics or antifungals. We collected newly-eclosed virgin females under CO_2, and allowed them to recover for seven days to minimise the physiological effects of anaesthesia (Nilson et al., 2006). Flies were exposed to cold in groups of 10 in food vials (3-15 vials per experiment/exposure/treatment) in aluminium blocks cooled from refrigerated circulators (c.f. Nyamukondiwa et al., 2011); flies were in darkness during the cold exposure, thus cold exposures were performed during the hours in which flies would usually experience darkness. We exposed groups of flies to acute (-2°C, 2h) or sustained (-0.5°C, 10h)

cold in vials with food to maintain high humidity. Controls were handled identically to their corresponding cold-exposed group, but maintained at 21.5°C. After cold exposure, we returned flies to rearing conditions for 2-6h [depending on the immune response to be measured, and based on previous (Zhang et al., 2011) or preliminary experiments], during which time all flies recover, and then measured immune activity.

2.2 Potential immunity

To determine the effect of cold exposure on potential immunity, we measured circulating haemocyte concentration (CHC) and phenoloxidase activity (PO) in haemolymph samples, and mRNA abundance of immune-related genes (IMD pathway: *attacin, cecropin, diptericin*; Toll pathway: *drosocin*, *drosomycin;* IMD and Toll: *defensin* and *metchnikowin;* Jak-STAT pathway*: Turandot-A* and *virus-induced RNA 1*). Statistical analyses were performed in SPSS. We used t-tests (CHC and mRNA abundance) or ANOVA (PO activity) to compare differences in potential immunity among treatments.

2.2.1 Circulating haemocyte counts

To estimate CHC, we collected haemolymph (n = 10 per treatment) following MacMillan and Hughson (2014), diluted haemolymph in anticoagulant buffer [0.55 % W/V cresyl violet, 0.5 % ethylene-diamine-tetraacetic acid in phosphate buffered saline (PBS)] and counted haemocytes in a Neubauer improved haemocytometer at 400× magnification.

2.2.2 PO activity

We collected haemolymph following MacMillan and Hughson (2014) and pooled haemolymph from 8-10 flies under oil, for a final volume of 0.1 µL (n = 3-5 pooled samples per treatment). We diluted the haemolymph in 10 µL of PBS, snap-froze the samples in liquid nitrogen, and stored them at – 80 °C until use. We measured PO activity spectrophotometrically using L-dihydroxyphenylalanine (L-DOPA; 4 mg/mL) as the substrate. We expressed phenoloxidase activity as change in absorbance at 492 nm /min / µL, obtained during the linear portion of the reaction (Wilson et al., 2001).

2.2.3 Measurement of relative mRNA abundance with qPCR

To investigate the expression of genes associated with Toll, IMD, and JAK/STAT pathways, we quantified the expression of genes related to products or intermediate components of pathways by quantitative real-time polymerase chain reaction (RT-qPCR) (Bing et al., 2012). For each cold exposure treatment, we snap-froze flies in liquid nitrogen 6 h following cold exposure (Zhang et al., 2011) and extracted and pooled RNA from 30 frozen flies per sample (n = 5 pooled samples per treatment) using Trizol Reagent. We dissolved the extracted RNA pellet in 60 µl RNasefree water and determined the total RNA concentration and purity ratio at 260 and 280 nm absorbance using a NanoDrop 2000 spectrophotometer and associated software. Only samples with a 260/280 ratio of 1.98 or more were used. To remove DNA contaminants, we treated the RNA samples with DNase. We mixed one microgram RNA from each sample with 1 µl of DNase I Amp Grade and 1 µl of 10X DNase I Reaction Buffer. After incubation at room temperature for 15 min, we added 1 µl of 25 mM EDTA incubated the samples at 65 °C for 5 min to inactivate the DNase. We cooled the samples on ice before using them for cDNA synthesis. To synthesize cDNA, we added 2 µl of oligodT, 4 µl qScript Flex Reaction Mix (5×)

and 1 µl qScript Reverse Transcriptase to 1 µg RNA of each sample. We then treated the samples at 42 °C for 75 min and 85 °C for 5 min. To amplify the cDNA, we used SYBR Green Master Mix. To normalize the data obtained from the target genes, we used *Rpl*-32 as a reference gene (Zaidman et al., 2011). To determine the efficiency of primers at different cDNA concentrations, we created standard curves of target genes and the reference gene using seven different concentrations of mixed cDNA samples (0, 4, 16, 64, 256, 1024 and 4096-fold dilution). We calculated threshold cycle (Ct) values using CFX Manager Software ver. 2.1 (Bio-Rad) and we calculated and normalised the expression ratio of target genes relative to controls. We standardised all values to *Rpl-32* as a housekeeping gene, and to controls using ΔΔCt (Pfaffl, 2001).

2.3 Realised immunity

To examine the effect of cold exposure on realised immunity, we measured survival after topical application of the fungus *Metarhizium anisopliae* (Le Bourg et al., 2009), wound-induced melanisation, and the ability to clear gram-positive (*Bacillus subtilis*) and gram-negative (*Escherichia coli*) bacteria from the haemolymph (McKean and Nunney, 2001). Flies were briefly anaesthetized with CO_2 prior to all measures. Statistical analyses were performed in R (R Development Core Team, 2010). We used a linear mixed-effects model (fungal infections; "vial" as a covariate), a general linear model (wounding response) and a three-way ANOVA (bacterial clearance) to compare differences in realised immunity among treatments.

2.3.1 Wounding response

Immediately after cold exposure, we pierced flies on the dorsal surface using a sterilized No. 000 insect pin (Figure S1). We photographed the pierced area of ten flies from each treatment group at 6, 12, and 24h post-cold-exposure. We then quantified melanisation by measuring the mean gray value of the pierced area using Image J software. To compare piercing-induced melanisation between cold-treated and control flies, we recorded the gray value of the pierced area of treated and control flies at specific time points (0, 6, and 12 h and after cold exposure). Darker colors with lower gray values indicated more piercing-induced melanisation.

2.3.2 Fungal infection

We obtained the broadly entomopathogenic fungus *Metarhizium anisopliae* (strain 2575, USDA, Ithaca, NY, USA) from Dr. Michael Bidochka (Brock University, St. Catharine's, ON, Canada). *Metarhizium anisopliae* is commonly isolated from temperate soils and the overwintering habitats of insects (Bidochka et al., 1998), and we performed preliminary experiments to ensure that the fungus was lethal to flies at their rearing temperature of 21.5 °C. Following 6 h of recovery from cold exposure, we used the method of Le Bourg et al. (2009) for infecting flies by shaking them on an agar plate (30 s) with sporulating fungus. Non-infected controls of each group were shaken on a sterile plate. We returned groups of flies to vials (n = 8-10 per vial; 5-7 vials per treatment) at 21.5 °C and monitored survival every 24 h for 16 d.

2.3.3 Bacterial clearance

To compare the ability of control and cold-exposed flies to clear bacteria from the haemolymph, we injected flies (n = 4-9 per infection, per treatment) with streptomycin-resistant gram-positive (*B. subtilis*) and streptomycin-resistant gram-negative (*E. coli*) bacteria suspended in PBS,

following (McKean and Nunney, 2001), with some modifications. We selected flies haphazardly from each treatment group and injected 105nL (~1×10^4 CFU) into the thorax through a glass capillary needle attached to a hydraulic manual microinjector (Sutter Instrument, Novato, CA, USA) 2 h or 6 h post-cold-exposure. We homogenized whole flies in 90µL PBS 30s post-injection (to confirm the concentration of injection), or after 5h or 12h recovery at 21.5°C. We diluted and plated 10µL of each homogenate in four replicated spots on Luria broth agar containing streptomycin (25 µg/mL), incubated the plates (37°C, 24h) and counted the number of colony-forming units.

3. Results

3.1 Potential immunity

Acute, but not sustained, cold, significantly increased CHC compared to controls (Fig. 1; Table 1). We did not detect any difference in PO activity among treatments ($F_{2,9} = 1.09$, $p = 0.38$). Expression of *diptericin* increased following acute cold, and expression of *Turandot-A* increased relative to controls following both acute and sustained exposures (Fig. 3; Table 1). Expression of *metchnikowin* was higher after acute than sustained cold exposure (Fig 3C; Table 1). No other changes in gene expression were observed (Table S2).

3.2 Realised immunity

Neither acute nor sustained cold changed the susceptibility of flies to fungal infection (Fig. 4; Table 1). Only one uninfected control died over the course of the experiment in both the acute and control treatments, and no mortality occurred in uninfected flies from the sustained cold treatment; we did not detect any significant difference in survival among uninfected controls

211 (sustained vs control: $t_9 = 0.92$, $p = 0.38$; acute vs control: $t_9 = 0.11$, $p = 0.91$). Wound-induced
212 cuticle darkening was unchanged by either treatment (Fig. 5; Table 1). Clearance of gram-
213 positive bacteria decreased in flies exposed to acute cold when measured within 2h of cold
214 exposure (Fig 6; Table 1), but bacterial-clearance ability recovered rapidly: following 6h of
215 recovery from cold, treatment did not affect clearance (Fig. 6; Table 1).
216

217 ## 4. Discussion

218 We explored the effects of acute and sustained cold exposure on immune activity in
219 *Drosophila melanogaster*. Acute cold exposure increased potential immunity (more circulating
220 haemocytes and increased expression of *diptericin* and *TurandotA*), but decreased one measure
221 of realised immunity (gram-positive bacterial clearance immediately after cold exposure).
222 Sustained cold exposure also increased *Turandot-A* expression but did not affect realised
223 immunity.

224

225 All cold-exposed flies were in chill coma (MacMillan et al., 2015), and these cold exposures
226 have measurable non-lethal fitness impacts (Marshall and Sinclair, 2010), as well as
227 transcriptomic profiles that suggest that they are both stressful exposures (Zhang et al., 2011).
228 However, because sustained cold exposure did not elicit an increase in immune activity, whereas
229 acute cold exposure increased potential immunity, it seems unlikely that cold-induced immune
230 activity is a by-product of a general stress response. Instead, we suggest that acute cold led to
231 increased cold shock injury [e.g. tissue damage associated with apoptosis (Yi et al., 2007) or
232 membrane phase transitions (Lee et al., 2006)] relative to the sustained exposure, which signaled
233 for an increase in potential immunity via direct signals from apoptotic or necrotic cells

234 (Maltzinger, 1998). For example, repeated cold exposure both increases tissue damage as well
235 as resistance to fungal infection in *Pyrrharctia isabella*, (Marshall and Sinclair, 2011),
236 suggesting that cold-induced immune activity is linked to cold injury. Because hemocytes are
237 responsible for the phagocytosis of apoptotic and damaged cells (Marmaras and Lampropoulou,
238 2009) and increase in circulating concentration following tissue damage (Pastor-Pareja et al.,
239 2008), increased CHC following acute cold could represent a direct immune response to chilling
240 injury.

241

242 Although acute cold exposure increased some measures of potential immunity, flies also
243 displayed a reduced ability to clear gram-positive bacteria, *B. subtilis,* shortly after acute cold
244 exposure (2h). This contrast between potential and realised responses suggests that the increase
245 in CHC and AMPs is concomitant with impairment of the immune system. If chilling injury
246 includes damage to the hemocytes themselves, the circulating hemocytes we counted may
247 include both functional and damaged cells (Yi and Lee, 2003); thus although more cells appear
248 to be in circulation, immune function is either impaired or unchanged. Additionally, recovery
249 from chilling injury is metabolically costly (MacMillan et al., 2012), as are immune responses
250 (Freitak et al., 2003), and the initial decrease in bacterial clearance after acute cold could
251 represent a trade-off between recovery from cold exposure and immune activity. For example,
252 infected *Drosophila* take longer to recover from cold exposure (Linderman et al., 2012),
253 suggesting that there is a conflict between the response to cold and the response to infection. In
254 either the case of direct damage or trade-offs between immunity and recovery from cold, cold
255 exposure appears to have the potential to impair immune activity.

Despite this initial impairment of immunity, activity appeared to have recovered by 6 h post cold exposure, we did not observe an impaired response to gram-negative *E. coli,* and nor was there a change in survival of fungal infection. The immune response (including rate of phagocytosis, the role of hemocytes, and the timing of expression of AMPs) varies depending on the type of pathogen encountered (Lemaitre and Hoffmann, 2007; Marmaras and Lampropoulou, 2009), thus it is possible that the mechanism for clearing *B. subtilis* is more affected by chilling injury than the mechanisms for responding to *E. coli* or *M. anispoliae*. Alternatively, up-regulation of *diptericin* through the IMD pathway (largely responsible for the response to gram-negative bacteria) may have compensated for impaired immunity and allowed the flies to maintain clearance of gram-negative *E. coli*. Similarly, recovery of the response to *B. subtilis* suggests that the immune response compensates for this initial, cold-induced impairment of activity.

We propose that the increase in potential immunity that we observed following acute cold exposure is a compensatory response to immune impairment through direct damage to the immune system or trades-off with the response to cold. This compensatory response may manifest as reconfiguration of the immune system (Adamo, 2014), that allows *Drosophila* to maintain responses to cold-induced tissue damage or pathogens in the environment. Overall, this suggests that cold-induced increases in immune activity are adaptive, and that it is important to maintain the ability to respond to pathogens following cold exposure. It will next be important to determine if conflicts between cold and immunity are present in other insect taxa, if they vary

seasonally, and whether they affect the fitness of overwintering insects experiencing both cold and pathogen stress.

In conclusion, we suggest that *Drosophila* have evolved immune activation by cold to maintain the ability to respond to immune challenge to compensate for cold-induced damage to, or trade-offs with, the immune system, but that this activation does not result in an increase in realised activity. In the context of multiple stressors, this therefore demonstrates an evolved resilience to the direct effect of an abiotic stressor on the ability to respond to pathogen stress, and provides (another) warning about the importance of comparing multiple measures of the immunity before drawing conclusions (Adamo, 2004).

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Figure Legends

Figure 1. Circulating haemocyte concentration of *Drosophila melanogaster* following cold exposure. Flies were exposed to acute (-2 °C, 2 h) or sustained (-0.5 °C, 10 h) cold; n=10 per treatment. Mean ± SEM is shown; asterisks indicate significant differences ($p<0.05$) between cold-exposed and control flies (see Table 1 for statistics).

Figure 2. Haemolymph phenoloxidase activity of *Drosophila melanogaster* following cold exposure. Flies were exposed to acute (-2 °C, 2 h) or sustained (-0.5 °C, 10 h) cold; n=3-5 per treatment (8-10 flies pooled per replicate). Mean ± SEM is shown.

Figure 3. Relative mRNA abundance of genes related to *Drosophila melanogaster* immune pathways measured by real-time qPCR, following cold exposure. Flies were exposed to acute (-2 °C, 2 h) or sustained (-0.5 °C, 10 h) cold; n=5/gene/treatment (30 flies pooled per replicate). *dros=drosomycin, def=defensin, mtk=metchnikowin, atta=attacin, cec=cecropin, dipt=diptericin, dro=drosocin, TotA=Turandot A* and *vir=vir-1*. Mean ± SEM is shown; asterisks indicate significant differences ($p<0.05$) between cold-exposed and control flies; different lower-case letters indicate significant differences between flies exposed to acute and sustained cold (see Table 1 for statistics).

Figure 4. Survival of cold-exposed *Drosophila melanogaster* infected with *Metarhizium anisopliae* (n=8-10 flies in 5-7 vials/treatment). Flies were exposed to acute (-2 °C, 2 h) or sustained (-0.5 °C, 10 h) cold and topically infected with *M. anispoliae* following recovery from cold. Mean ± SEM is shown (see Table 1 for statistics).

Figure 5. Wounding response in *Drosophila melanogaster* measured as cuticle melanisation (grey value) following cold exposure. Flies were exposed to acute (-2 °C, 2 h) or sustained (-0.5 °C, 10 h) cold and pierced in the thorax with an insect pin following recovery from cold. n = 10 per treatment, per time point (see Table 1 for statistics).

Figure 6. *In vivo* clearance of *Escherichia coli* and *Bacillus subtilis* from the hemolymph of *Drosophila melanogaster* following cold exposure. Flies were exposed to acute (-2 °C, 2 h) or sustained (-0.5 °C, 10 h) cold and injected with a suspension of bacteria 2 h or 6 h following recovery from cold. The proportion of bacteria cleared from the hemolymph was assessed either 5 h or 12 h following injection; (n=4-9/treatment/infection). Lower-case letters indicate significant differences ($p<0.05$) between acute cold-exposed, sustained cold-exposed, and control flies (see Table 1 for statistics).

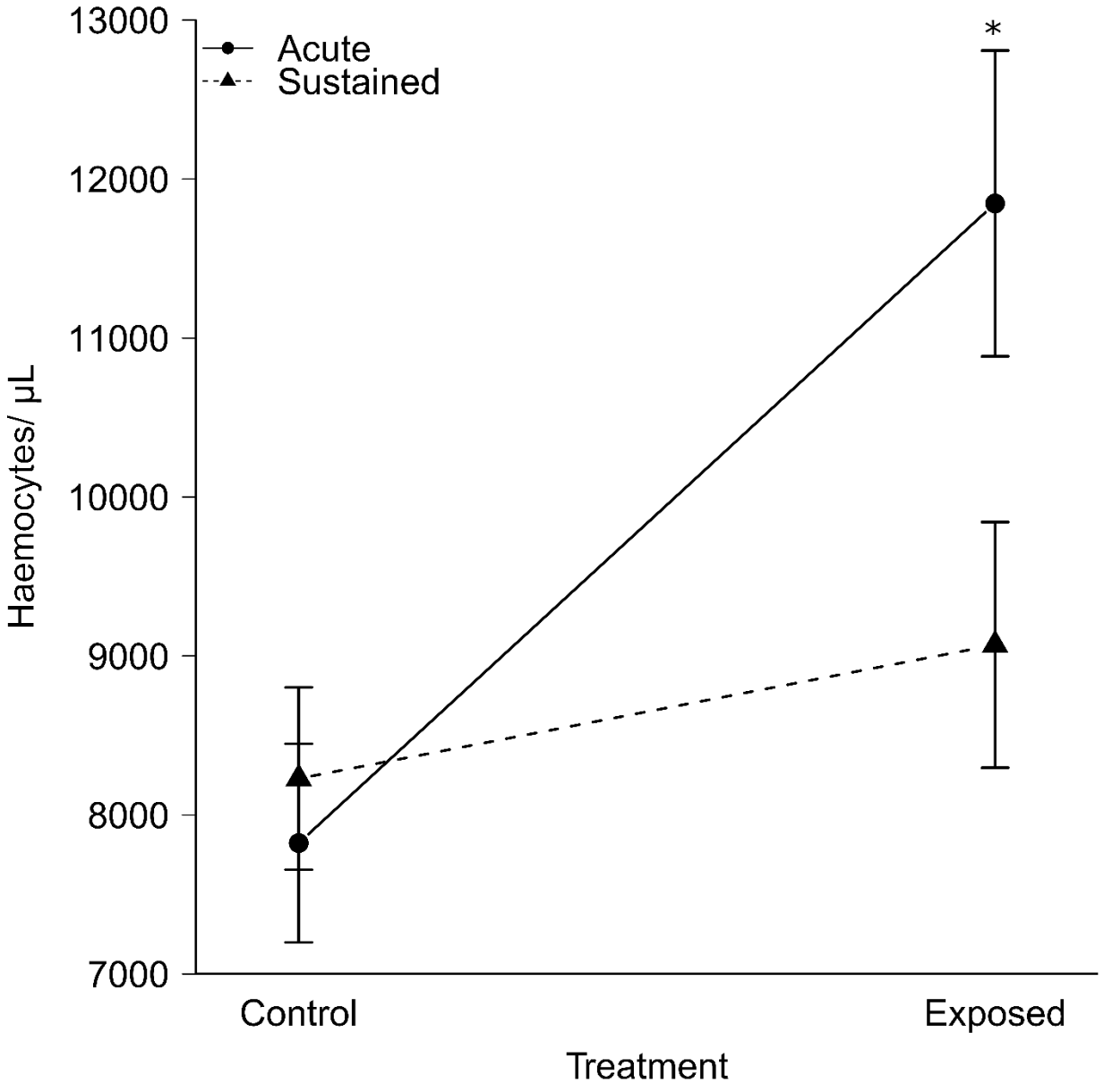
Table 1. Effects of acute (2 h, -2 °C) or sustained (10 h, -0.5 °C) cold exposure on potential and realised immunity of female *Drosophila melanogaster*. Cold refers to both acute and sustained exposures, compared to controls. P-values in bold indicate significant differences. PC = post-cold exposure; PI = post-infection

Measure	Term(s)	Statistic	p
Potential immunity			
Circulating haemocyte counts	Acute vs control	$t_{15} = 3.51$	**<0.05**
	Sustained vs control	$t_{16} = 0.87$	0.39
mRNA abundance	Acute vs control (*diptericin*)	$t_4 = 7.10$	**<0.01**
	Acute vs control (*Turandot-A*)	$t_4 = 3.43$	**<0.001**
	Sustained vs control (*Turandot-A*)	$t_4 = 11.95$	**<0.001**
	Acute vs sustained (*Metchnikowin*)	$t_4 = 8.54$	**0.046**
Realised immunity			
Fungal infection	Infected vs uninfected	$t_{252} = 34.37$	**<0.001**
	Acute vs control	$t_{14} = 1.17$	0.26
	Sustained vs control	$t_{14} = 1.72$	0.11
Melanisation	Acute vs control	$t_{90} = 1.76$	0.08
	Sustained vs control	$t_{90} = 1.21$	0.23
Bacterial clearance			
2 h PC; 5 h PI	Treatment	$F_{2,23} = 9.10$	**<0.01**
	Bacteria	$F_{1,23} = 13.26$	**<0.001**
	Treatment × Bacteria	$F_{2,23} = 3.37$	0.067
6 h PC, 5 h PI	Treatment	$F_{2,22} = 0.50$	0.61
	Bacteria	$F_{1,22} = 7.82$	**0.01**
	Treatment × Bacteria	$F_{2,22} = 0.93$	0.93
6 h PC; 12 h PI	Treatment	$F_{2,17} = 0.89$	0.92
	Bacteria	$F_{1,17} = 0.511$	**0.03**
	Treatment × Bacteria	$F_{2,17} = 0.892$	0.43

421

422

423 Figure 1

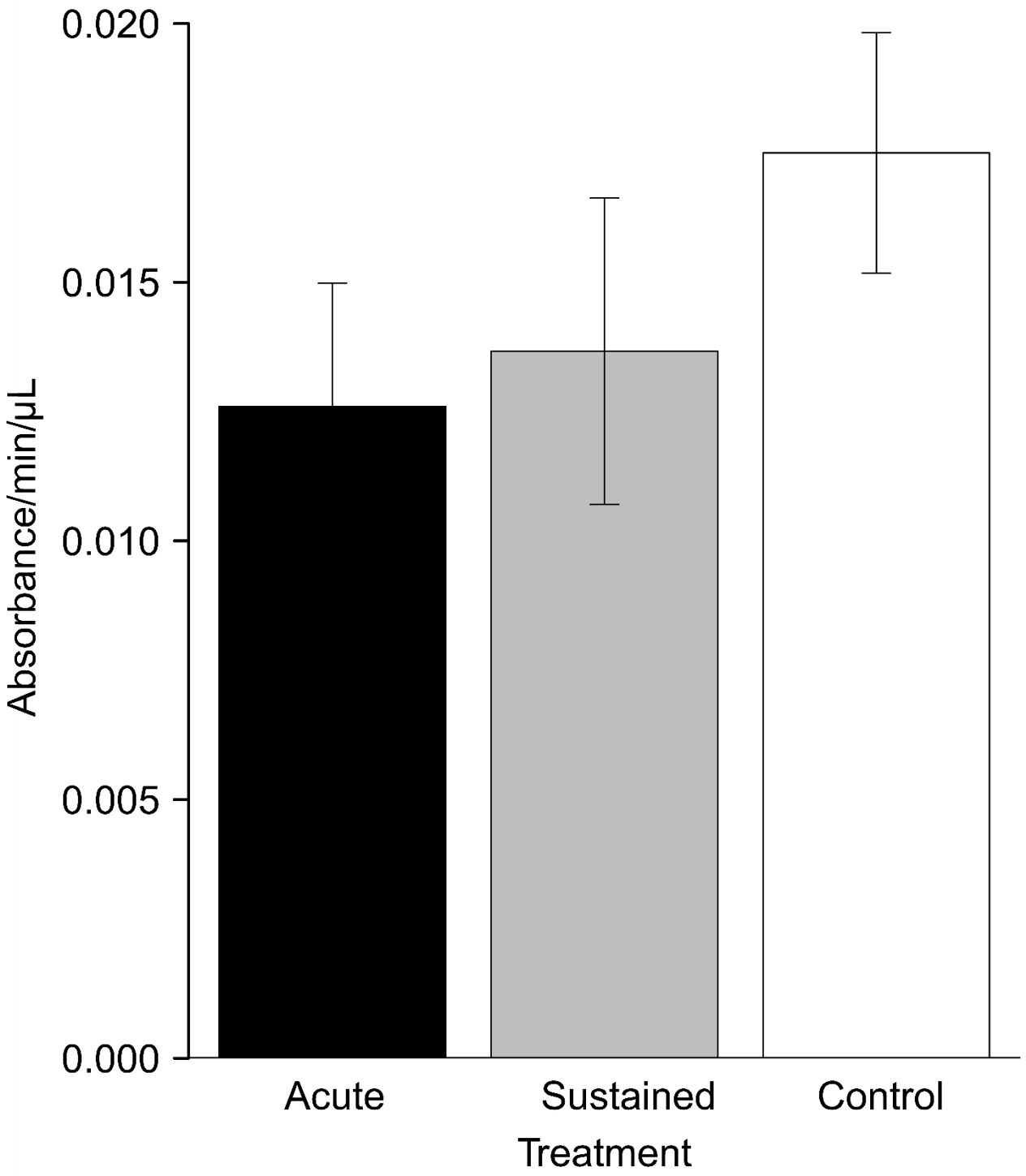


424

425

426 Figure 2

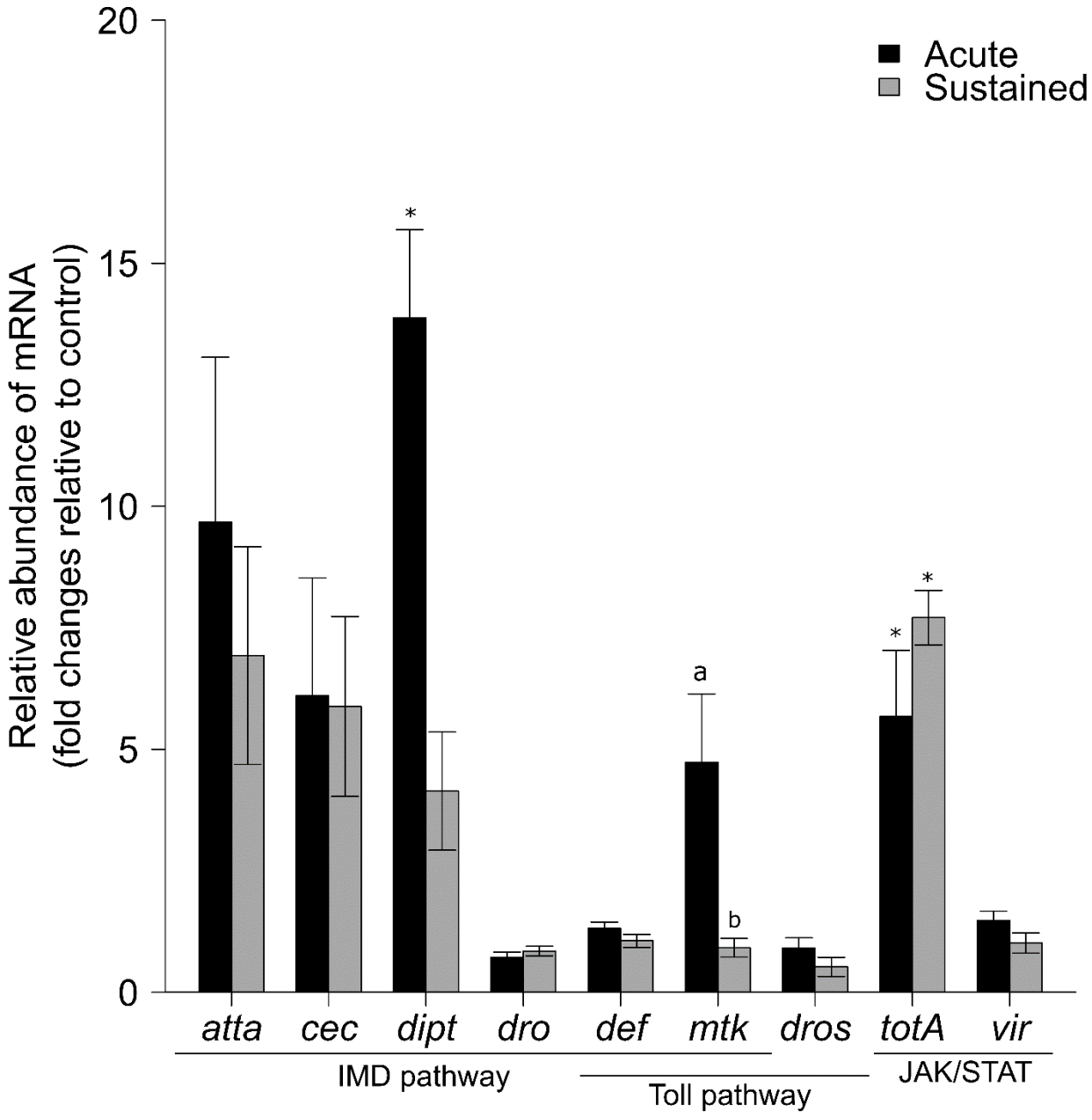
427



428

429 Figure 3

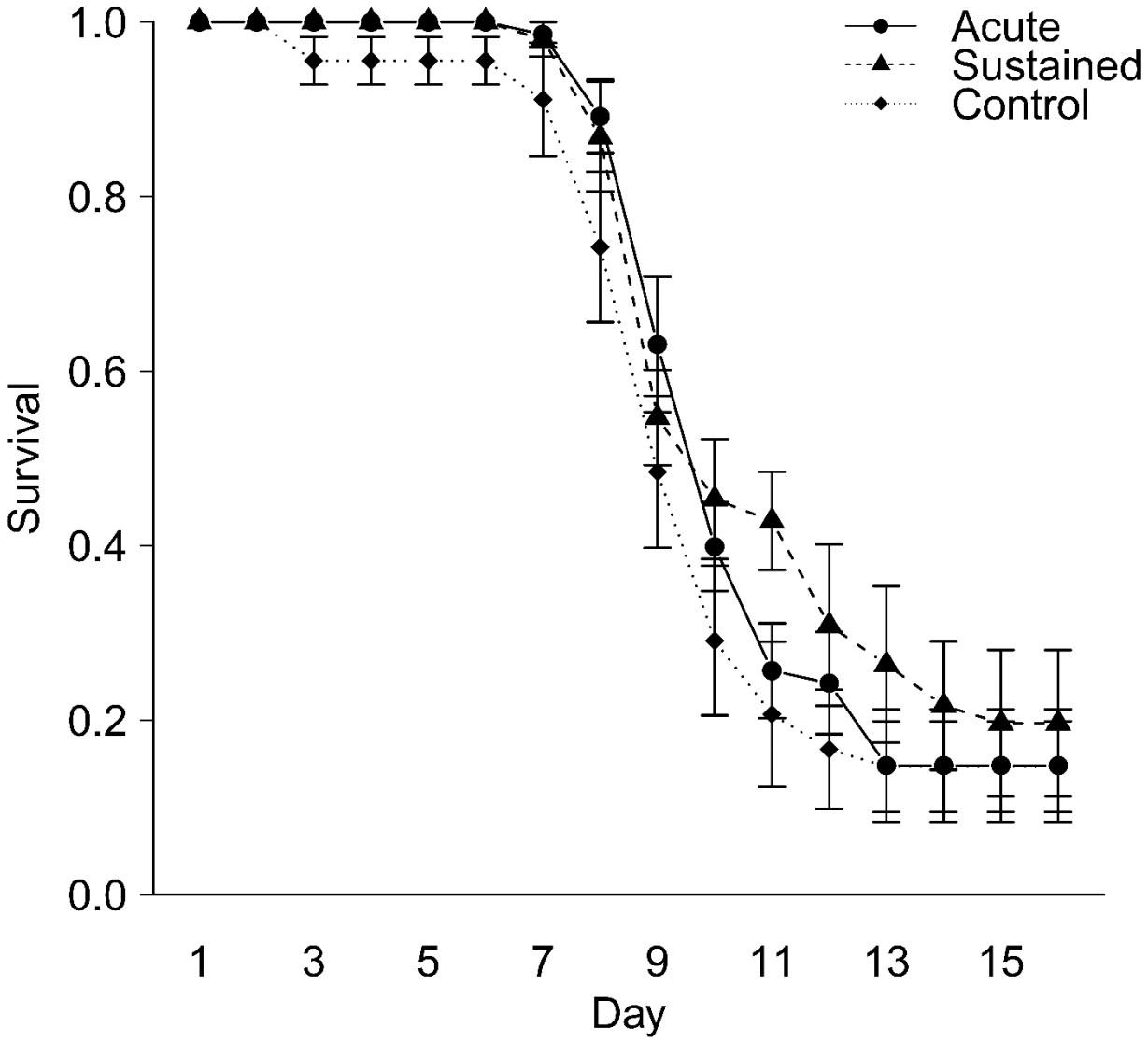
430



431

433 Figure 4

434



435

436

437 Figure 5

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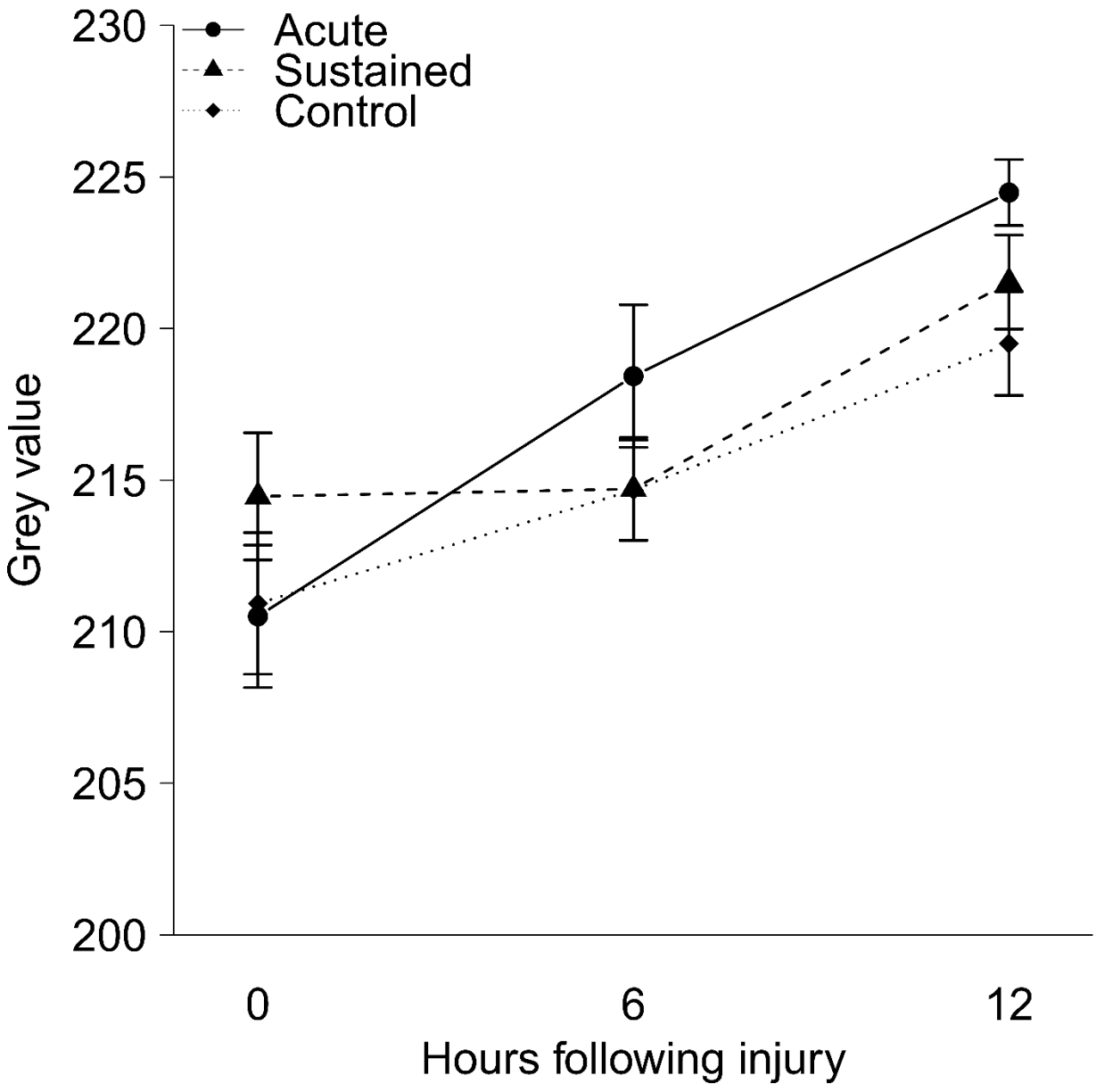
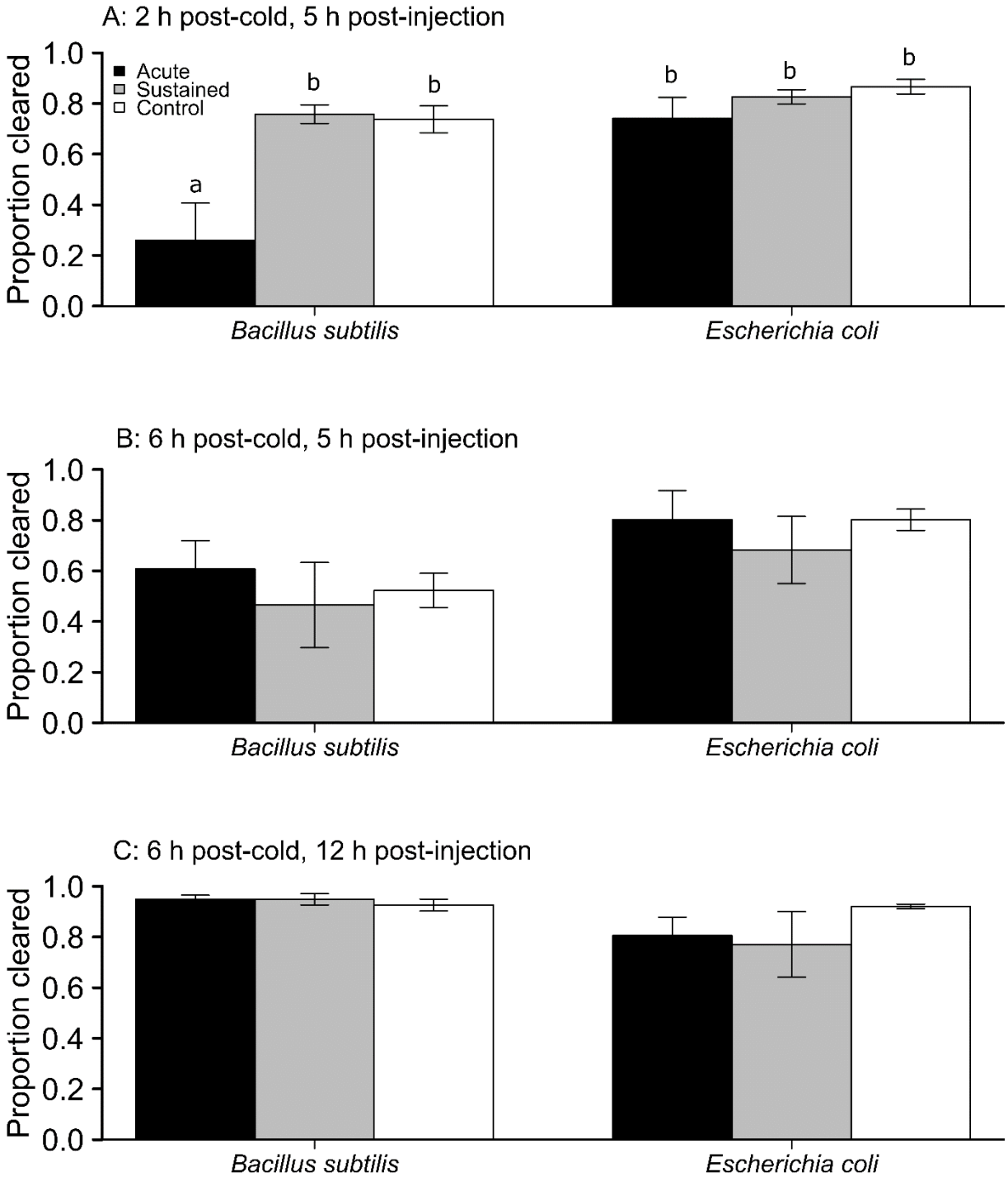


439

440

441 Figure 6

442



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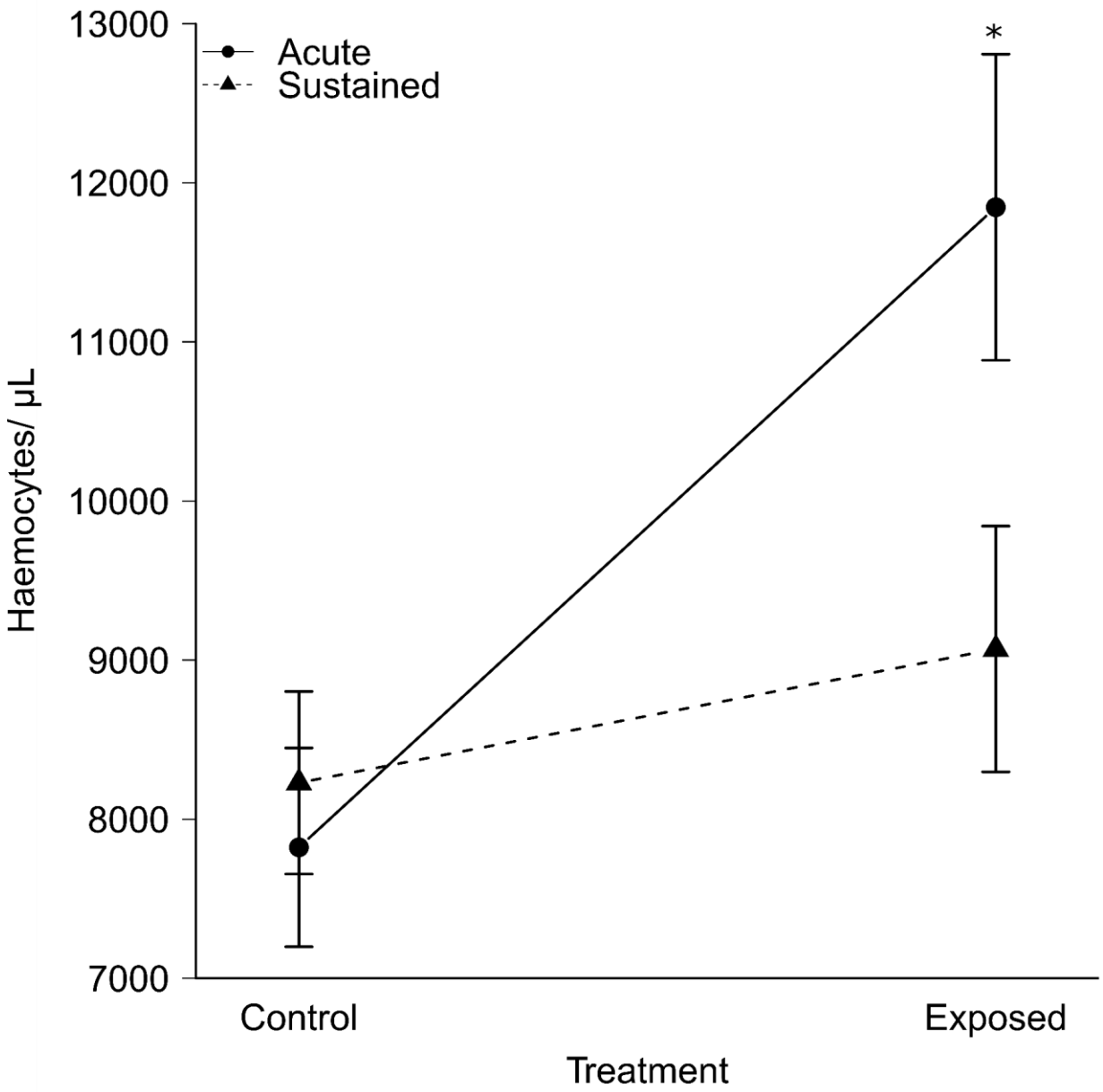
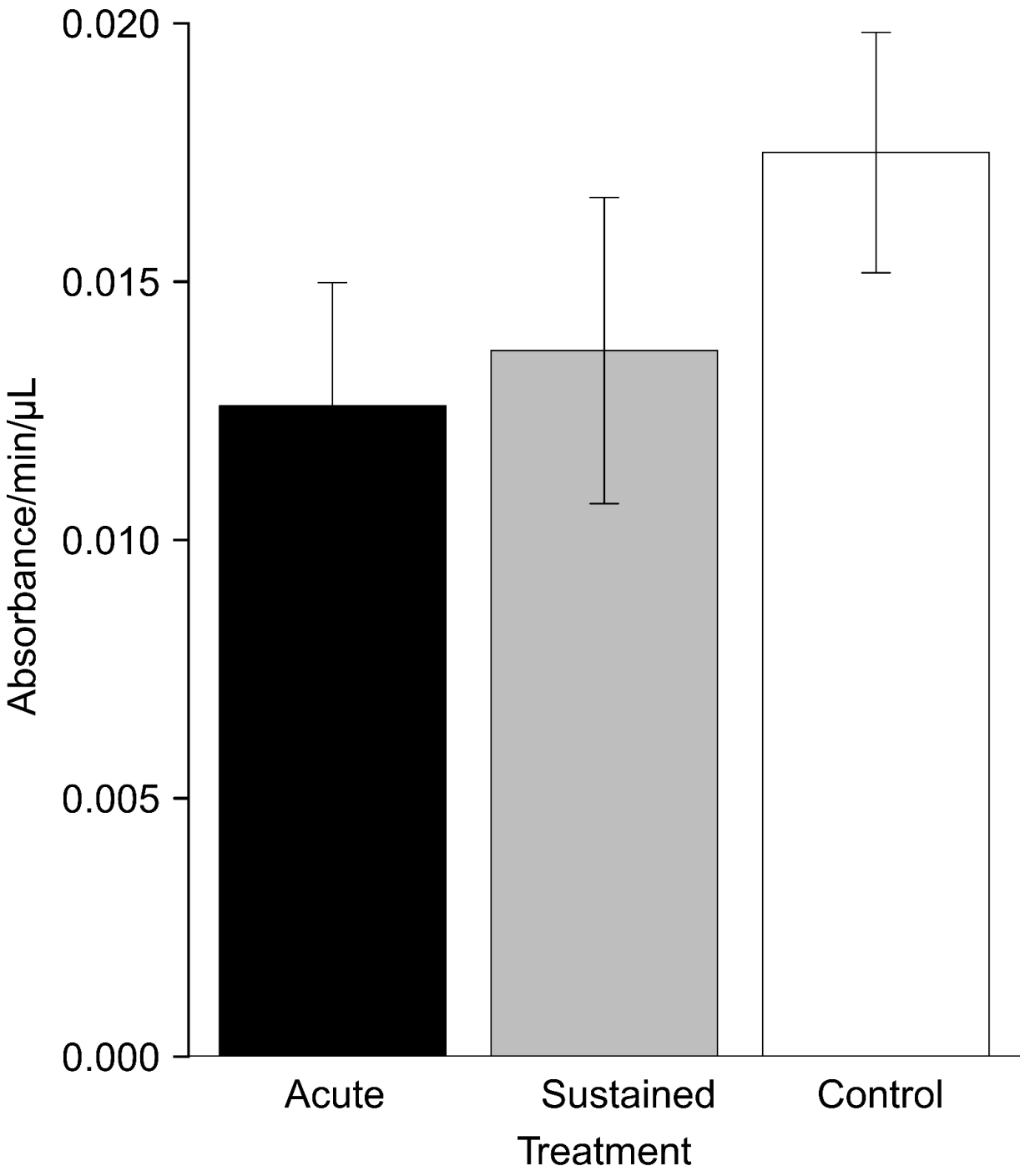
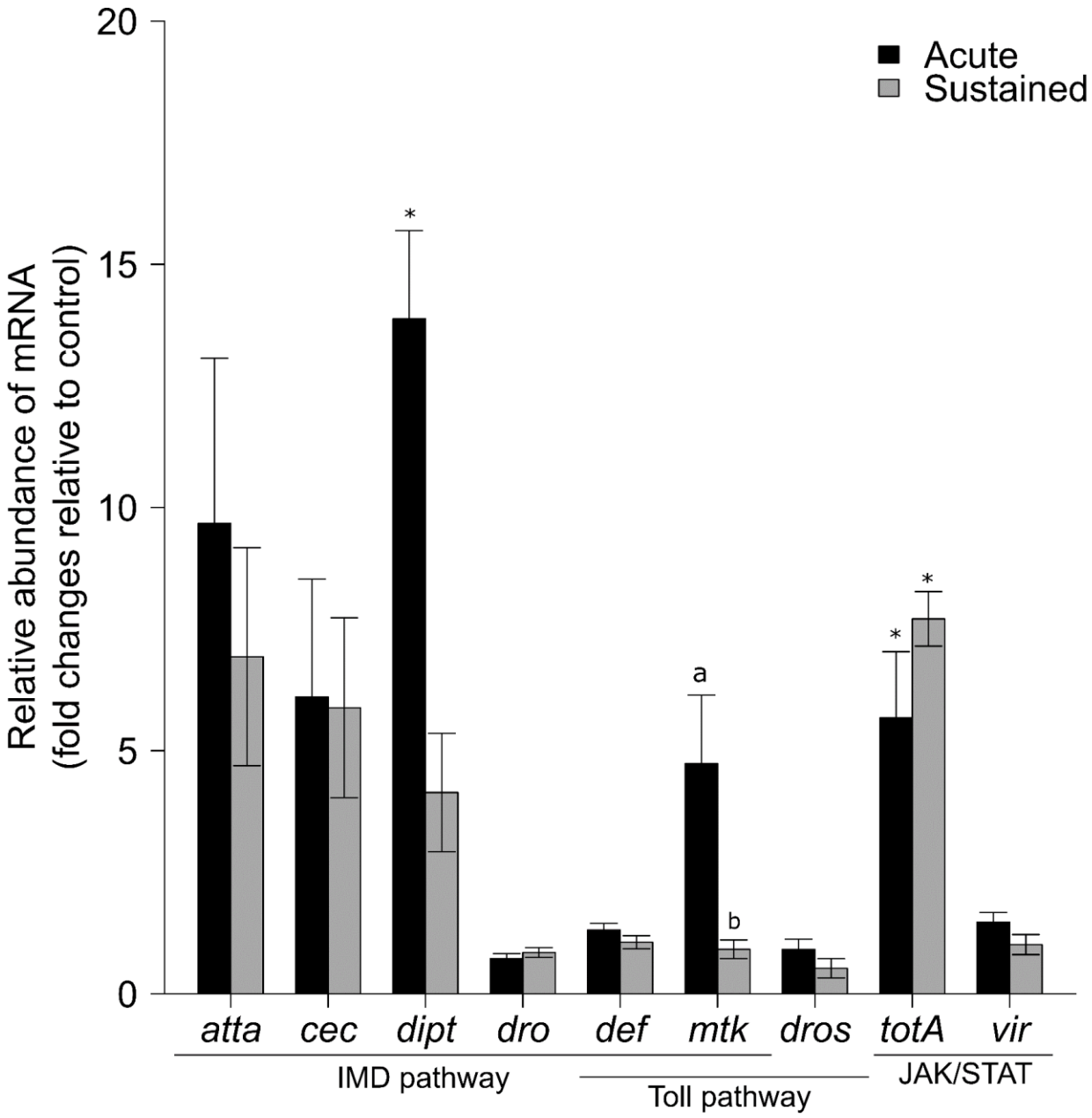
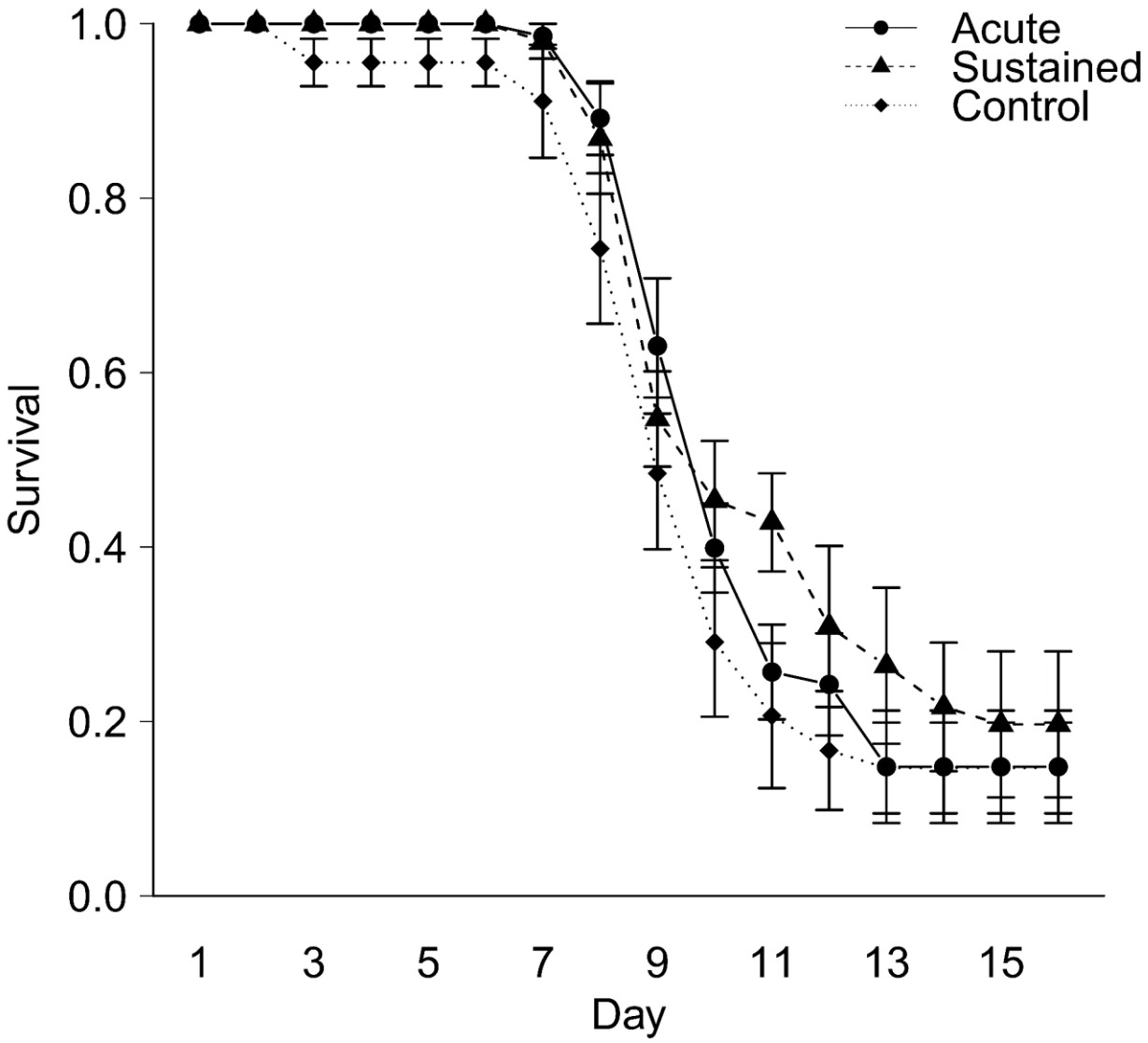
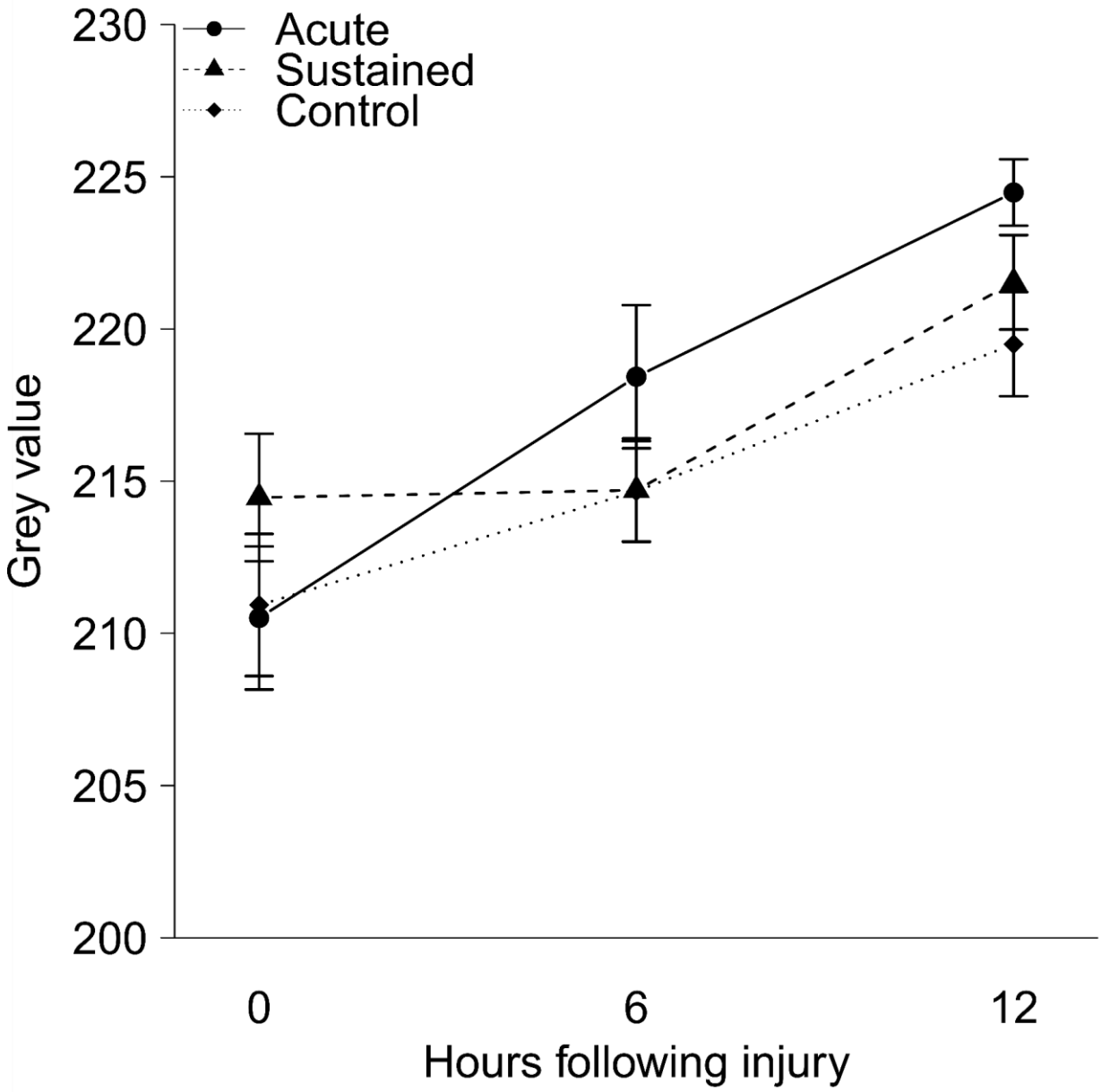
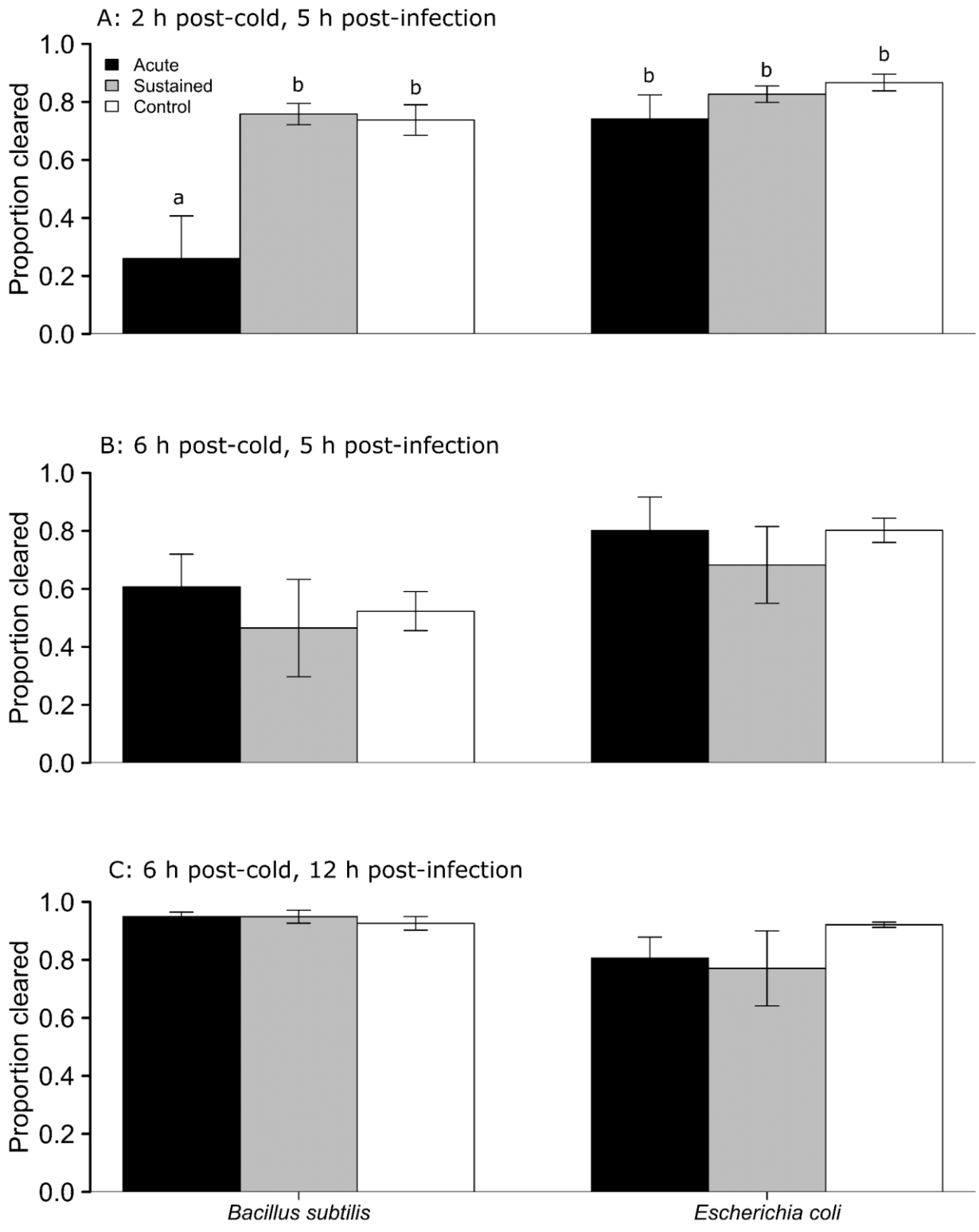

13000
12000
11000
10000
9000
8000
7000
Haemocytes/ µL
Acute
Sustained
*
Control
Exposed
Treatment


0.020
0.015
0.010
0.005
0.000
Absorbance/min/µL
Acute
Sustained
Control
Treatment


Acute
Sustained
Relative abundance of mRNA
(fold changes relative to control)
20
15
10
5
0
*
a
b
*
*
atta
cec
dipt
dro
def
mtk
dros
totA
vir
IMD pathway
Toll pathway
JAK/STAT


Acute
Sustained
Control
Survival
1.0
0.8
0.6
0.4
0.2
0.0
1
3
5
7
9
11
13
15
Day


Acute
Sustained
Control
230
225
220
215
210
205
200
Grey value
0
6
12
Hours following injury


A: 2 h post-cold, 5 h post-infection
Acute
Sustained
Control
Proportion cleared
1.0
0.8
0.6
0.4
0.2
0.0
a
b
b
b
b
b
B: 6 h post-cold, 5 h post-infection
Proportion cleared
1.0
0.8
0.6
0.4
0.2
0.0
C: 6 h post-cold, 12 h post-infection
Proportion cleared
1.0
0.8
0.6
0.4
0.2
0.0
Bacillus subtilis
Escherichia coli

Supplemental methods and results

Table S1: Primers used to measure expression of genes related to Toll, IMD, and Jak/STAT pathways in *Drosophila melanogaster* using q-PCR. The reference gene is Rpl32. T_m indicates the melting temperature. References are noted for primers derived from literature. When designed in-house, Primer3 was used to design primers with amplicons of less than 250 bp long and GC content of 45-65% with no self-complementarity. Amplification efficiency of the primers was checked to be more than 95% using calibration curves of serially diluted concentration of the primers and the specificity of the primers was verified by PCR.

Gene	Nucleotide sequence (5' to 3')	T_m	Reference
Rpl32	5'-GACGCTTCAAGGGACAGTATCTG-3'	62	[1]
	5'-AAACGCGGTTCTGCATGAG-3'	62	
attacin-B	5'-GGCCCATGCCAATTTATTCA-3'	63	[2]
	5'-CATTGCGCTGGAACTCGAA-3'	63	
cecropin-A	5'-TCTTCGTTTTCGTCGCTCTC-3'	61	[2]
	5'-CTTGTTGAGCGATTCCCAGT-3'	60	
defensin	5'-GCCAGAAGCGAGCCACAT-3'	63	[2]
	5'-CGGTGTGGTTCCAGTTCCA-3'	63	
diptericin-A	5'-AGGTGTGGACCAGCGACAA-3'	63	[2]
	5'-TGCTGTCCATATCCTCCATTCA-3'	63	
drosocin	5'-CCACCACTCCAAGCACAATG-3'	60	
	5'-TGAGTCAGGTGATCCTCGATGG-3'	58	
drosomycin-B	5'-CTCCGTGAGAACCTTTTCCA-3'	60	[2]
	5'-GTATCTTCCGGACAGGCAGT-3'	59	
metchnikowin	5'- CTACATCAGTGCTGGCAGAG-3'	60	
	5'- CGGTCTTGGTTGGTTAGGATTG-3'	58	
PGRP-LB	5'-TGTGGCCGCTTTAGTGCTT-3'	62	[2]
	5'-TCAATCTGCAGGGCATTGG-3'	63	
PGRP-LC	5'-ACGGAATCCAAGCGTATCAG-3'	60	[2]
	5'-GGCCTCCGAATCACTATCAA-3'	60	
PGRP-SB	5'-CTGCGGCTGTTATCAGTGAA-3'	60	[2]
	5'-TGATGGAATTTCCGCTTTTC-3'	60	
PGRP-SD	5'-CCTTGCCACGTGCTGTGA-3'	63	[2]
	5'-TGTAACATCATCCGCACAAGCT-3'	63	
relish	5'-GTGGAGTTGGACCTAAGTAGTGG-3'	55 59	
	5'- TGATTCAGCAGCGAACAGAGC-3'		
toll	5'-AACTTGGGCAACCTTGTGAC-3'	60	[2]
	5'-GTAACCAAACGGGGAGTTGA-3'	60	
TotA-1	5'-TGAGGAACGGGAGAGTATCG-3'	60	[2]
	5'-GCCCTTCACACCTGGAGATA-3'	60	
vir-1	5'-TGTGCCCATTGACCTATCCA-3'	62	[2]
	5'-GATTACAGCTGGGTGCACAA-3'	60	

Table S2. Statistical analyses of the effect of acute and chronic cold on expression level of immune genes of *Drosophila melanogaster* females. The data shows the relative abundance of the immune genes mRNA (normalized to Rpl-32) after acute cold exposure (2h, -2 °C) and chronic cold exposure (10h, -0.5 °C).

Gene	Treatment	Test value	*P*-value	Adjusted *P*
attacin-B	Acute	2.56	0.063	0.13
	Chronic	2.65	0.057	0.13
cecropin-A	Acute	2.11	0.102	0.13
	Chronic	2.64	0.058	0.14
defensin	Acute	2.35	0.078	0.13
	Chronic	0.46	0.67	0.75
diptericin-A	Acute	7.10	<0.01	0.09
	Chronic	2.578	0.061	0.13
drosocin	Acute	2.66	0.056	0.13
	Chronic	1.66	0.172	0.22
drosomycin-B	Acute	0.41	0.701	0.75
	Chronic	2.42	0.072	0.13
metchnikowin	Acute	2.66	0.046	0.13
	Chronic	0.33	0.756	0.76
PGRP-LB	Acute	2.47	0.069	0.13
	Chronic	2.47	0.070	0.13
PGRP-LC	Acute	2.60	0.060	0.13
	Chronic	1.69	0.166	0.22
PGRP-SB	Acute	2.26	0.086	0.13
	Chronic	2.59	0.060	0.13
PGRP-SD	Acute	1.46	0.22	0.26
	Chronic	0.32	0.76	0.76
relish	Acute	2.44	0.071	0.13
	Chronic	2.57	0.061	0.13
toll	Acute	1.05	0.352	0.40
	Chronic	1.45	0.220	0.26
TotA-1	Acute	3.43	<0.001	0.01
	Chronic	11.59	<0.001	0.01
vir-1	Acute	2.40	0.074	0.13
	Chronic	0.06	0.0956	0.14

Figure S1. Location of piercing for wound-induced melanisation. Flies were pierced in the right thoracic vertical cleft along the notopleural suture until it reached the humeral callus, as indicated by the black arrow.