Drosophila macrophages switch to aerobic glycolysis to mount effective antibacterial defense

Gabriela Krejčová*1, Adéla Danielová1, Pavla Nedbalová1, Michalina Kazek1, Lukáš Strych1, Geetanjali Chawla2#, Jason M. Tennessen2, Jaroslava Lieskovská3,4, Marek Jindra1,5, Tomáš Doležal1, and Adam Bajgar*1,5

*First author

*Corresponding authors

1 Department of Molecular Biology and Genetics, University of South Bohemia in Ceske Budejovice, Czech Republic.

2 Department of Biology, Indiana University, Bloomington, IN 47405, United States of America.

#Present address: Regional Centre for Biotechnology, Faridabad, India.

3 Department of Medical Biology, University of South Bohemia in Ceske Budejovice, Czech Republic.

4 Institute of Parasitology, Biology Centre CAS, Ceske Budejovice, Czech Republic.

5 Institute of Entomology, Biology Centre CAS, Ceske Budejovice, Czech Republic.
**Abstract**

Macrophage-mediated phagocytosis and cytokine production represent the front lines of resistance to bacterial invaders. A key feature of this pro-inflammatory response in mammals is the complex remodeling of cellular metabolism towards aerobic glycolysis. Although, the function of bactericidal macrophages is highly conserved, the metabolic remodeling of insect macrophages remains poorly understood. Here we used the adult fruit fly *Drosophila melanogaster* to investigate the metabolic changes that occur in macrophages during the acute and resolution phases of *Streptococcus*-induced sepsis. Our studies revealed that orthologs of the Hypoxia inducible factor 1α (HIF1α) and Lactate dehydrogenase (LDH) are required for macrophage activation, their bactericidal function, and resistance to infection, thus documenting conservation of this cellular response between insect and mammals. Further, we show that macrophages employing aerobic glycolysis induce changes in systemic metabolism that are necessary to meet the biosynthetic and energetic demands of their function and resistance to bacterial infection.

**Introduction**

Macrophages represent a highly specialized and versatile population of cells that occur in all animals and perform a diversity of functions [1]. In the absence of an activating stimulus, macrophages reside as quiescent sentinel cells with minimal metabolic requirements [2]. In response to extracellular triggers, however, macrophages undergo a dramatic change in behavior that coincides with an enhanced metabolic rate and increased energy demands [3]. In this regard, the manner by which macrophages mount a response is dictated by the activating stimuli, which include tissue damage (DAMPs), pathogen (PAMPs) or microbe (MAMPs) associated molecular patterns, and signaling molecules that are secreted by other cells, such as cytokines. Each challenge requires induction of
specific metabolic and physiological processes that allow for an adequate immune response [4] – cellular changes that are collectively known as a polarization phenotype.

Macrophages polarize into bactericidal (M1) or healing (M2) functional phenotypes characterized mainly by metabolism [5]. M1 and M2 polarization phenotypes utilize distinct ways of ATP generation (glycolysis vs. oxidative phosphorylation) and metabolism of arginine (NO synthesis vs. ornithine cycle) [6]. Nowadays, the whole spectrum of polarization phenotypes corresponding to particular functions has been described [7], [8]. Perhaps the most dramatic change in macrophage metabolism associates with the M1 bactericidal phenotype, where cells increase both glucose consumption and lactate production independent of oxygen concentration - a phenomenon known as aerobic glycolysis (AG) [9], [10]. The resulting metabolic program promotes increased glucose catabolism, thus allowing M1 macrophages to generate enough of the ATP and glycolytic intermediates necessary for elevated phagocytic cell activity [11]. This shift in cellular metabolism towards AG appears to be a determining factor in macrophage function and development of the pro-inflammatory phenotype [12].

A key regulator of AG within macrophages is Hypoxia inducible factor 1α (HIF1α). While this transcription factor is normally degraded in the presence of oxygen, the triggering of either Toll-like receptor (TLR) or Tumor necrosis factor receptor (TNFR) signaling within macrophages activates Nuclear factor kappa-B (NFκB) and stabilizes HIF1α independent of oxygen availability [13], [14]. This normoxic HIF1α stabilization promotes the expression of genes under the control of hypoxia response elements (HREs), many of which are involved in cellular metabolism, cell survival, proliferation, and cytokine signaling [15]. In this regard, two of the key HIF1α target genes encode the enzymes Pyruvate dehydrogenase kinase (PDK) and Lactate dehydrogenase (LDH), which together shunt pyruvate away from the mitochondria and maintain NAD⁺/NADH redox balance independent of oxidative phosphorylation. Inhibition of both HIF1α and LDH represents efficient strategy to experimentally direct
cellular metabolism from AG to oxidative phosphorylation in both mice and Drosophila [16], [17], demonstrating their crucial role in this metabolic switch.

Although pyruvate metabolism within the tricarboxylic acid (TCA) cycle is limited during AG, the TCA intermediates are essential for many cellular processes. Therefore, cells under AG rely on feeding the TCA cycle with glutamine, thus causing a TCA cycle to be “broken” [18]. Such a dramatic change of in mitochondrial metabolism leads to significant imbalances in the cytosolic accumulation of TCA metabolites (such as NO, succinate, fumarate, L-2-hydroxyglutarate) that further contribute to HIF1α stabilization [19]. While this feedback maintains AG, at the same time it makes it dependent on sufficient supply of nutrients from the environment [20].

Macrophages employing AG must consume sufficient carbohydrates to support biosynthesis and growth. In order to ensure an adequate supply of sugar and other nutrients, these cells produce signaling molecules that affect systemic metabolism in order to secure enough energy for themselves – a concept recently defined as selfish immune theory [21] [22]. According to this theory, signaling molecules released by immune cells induce systemic metabolic changes such as hyperglycemia and systemic insulin resistance to increase titer of nutrients to be available for the immune response and to limit their consumption by other tissues and processes [23]. Since many of these signaling molecules are direct HIF1α transcriptional targets, HIF1α stabilization directs the cellular metabolism while it simultaneously induces expression of genes with an impact on the whole systemic metabolism [24], [25]. Thus, macrophages are not just phagocytosing cells but they also regulate the systemic metabolism of an organism.

Similar to mammals, Drosophila macrophages serve an essential role in the immune system and are capable of responding to a wide array of stimuli, ranging from pathogenic bacteria and fungi to the corpses of apoptotic cells [26], [27], [28]. The mechanism of the bactericidal function itself is highly
conserved at the molecular level between *Drosophila* and mammalian macrophages, including two central signaling pathways, Toll and Imd (TLR and TNFR functional homologs), which are triggered in response to pathogenic stimuli [29], [30], [31]. Since Toll and Imd pathways induce the NFκB signaling in *Drosophila*, we can assume that phagocytic role of macrophages may be accompanied by stabilization of the HIF1α ortholog, *Similar (Sima)*, hereafter referred to as Hif1α [32]. Indeed, normoxic stabilization of Hif1α followed by its nuclear localization and increased expression of HRE-controlled genes can induce metabolic changes typical for AG [33], [34], [35], [36], [37]. Even though the HRE-controlled genes frequently appear in transcriptomic data of activated insect macrophages [38], [39], the direct role of Hif1α in the macrophages has not yet been tested.

Considering that the molecular mechanisms controlling macrophage activation are similar in both *Drosophila* and humans, it seems logical that the metabolic changes occurring within these cells would also be comparable, however, the metabolism of insect macrophages remains poorly understood. Here we address this question by analyzing *in vivo* metabolic and transcriptional changes of adult *Drosophila* phagocytic macrophages by employing a model of *Streptococcus pneumoniae*-induced sepsis. The well-defined progress of this infection allowed us to distinguish three phases of the immune response according to the changing dynamics of bacterial growth (acute, plateau, and resolution phase) (Figure 1A). The acute phase lasts for the first 24 hours during which streptococcal population is rapidly growing, and its abundance must be limited by phagocytosis to avert early death [40] [41]. The established equilibrium between continuous bacterial growth and host bacterial killing results in the plateau phase lasting for the next four days. At the end of this period, the immune system of survivors surmounts the infection and clears the majority of the pathogens. The following resolution phase (120 hours post-infection (hpi) and later), is essential for macrophage-mediated clearance of bacterial residues and reestablishment of homeostasis [41] [42].
To analyze processes characteristic of highly active phagocytic macrophages in *Drosophila*, we compared attributes of acute phase macrophages (APMФs) with macrophages from uninfected individuals and resolution phase macrophages (RPMФs). Using a previously described hemolectin-driven GFP (*HmlGal4*>UAS-*eGFP*) [43], we isolated *Drosophila* adult macrophages (approximately 15 000 cells/replicate) and analyzed the metabolic and transcriptional responses that are induced within these cells upon infection (Figure 1B). Our approach revealed that *Drosophila* macrophages respond to the acute phase of bacterial infection by increasing glucose uptake, elevating glycolytic flux, and producing lactate. Moreover, similar to mammals, the activation and maintenance of AG within *Drosophila* macrophages depend on Hif1α, and requires elevated Ldh activity. We also demonstrate that induction of AG within *Drosophila* macrophages leads to change in systemic carbohydrate metabolism. Overall, our findings demonstrate that *Drosophila* macrophages must induce both autonomous and systemic changes in carbohydrate metabolism to mount a proper bactericidal function and resist infection.

**Results**

*Drosophila* macrophages undergo a metabolic shift to aerobic glycolysis during the acute phase of bacterial infection

Since the bactericidal function of phagocytic cells is connected with AG in mice [5], we analyzed *Drosophila* macrophages for the occurrence of AG hallmarks, such as increased glucose uptake, rise of the glycolytic flux, and generation of NADH pool facilitating Ldh-mediated reduction of pyruvate to lactate [18]. Distribution of fluorescently-labeled deoxyglucose (NBDG) in an organism, frequently used in cancer research, reflects the competitive potential of tissues in glucose internalization [44]. We tested the effect of immune response activation on glucose distribution among tissues in *Drosophila* by feeding the infected or control flies with NBDG during a 24-hour period before the signal detection. Infected
flies displayed prominent NBDG accumulation in APMΦs compared to other tissues, which is in contrast to uninfected controls or flies fed during the resolution phase of infection that displayed no such accumulation (Figure 2A, B). These results indicate an increased potential of phagocytosing macrophages to consume glucose in direct competition with other tissues during the acute phase of bacterial infection.

The increased NBDG uptake by macrophages was further supported by gene expression analysis, which revealed that the transcription of genes encoding both glycolytic enzymes and LDH, but not TCA cycle enzymes, were significantly up-regulated in APMΦs (Figure 2C). Moreover, these changes in glycolytic genes were restricted to the acute phase of infection as most glycolytic genes returned to a basal level of expression during the resolution phase, while hexokinase and enolase showed even a decreased expression similarly to all analyzed TCA cycle genes (Figure 2C and Figure 2 - figure supplement 1), which can be ascribed to global suppression of metabolism in these cells. Overall, these results indicate that macrophages specifically up-regulate glucose metabolism in response to S. pneumoniae infection.

Increased glucose uptake and expression of glycolytic genes, including Ldh, suggest an increased glycolytic flux and preferential reduction of pyruvate to lactate in APMΦs. To confirm this, we measured the enzymatic activity of LDH as an enzyme responsible for the diversion of pyruvate from TCA and Phosphoglucone isomerase (Pgi) as a glycolytic enzyme representative. In agreement with the expression data, Pgi enzymatic activity was significantly increased in APMΦs compared to control and compared to the situation observed during the resolution phase of infection (Figure 2D). The activity of Ldh increased not only in APMΦs but also in RPMΦs (Figure 2E). Moreover, the observed increase in Ldh activity was directly correlated with increased lactate production in vivo, as the hemolymph of infected individuals during both the acute and resolution phases of infection contained significantly elevated
lactate levels as compared to controls (Figure 2G). Overall, our results demonstrate that Drosophila macrophages respond to S. pneumoniae infection by up-regulating lactate production.

The primary reason why cells produce lactate as a byproduct of AG is to maintain NAD+/NADH redox balance. High levels of glycolytic flux produce excess NADH as a result of Glyceraldehyde-3-phosphate dehydrogenase 1 activity (Gapdh1) [45]. Consistently we observed that NADH levels were significantly increased in APMΦs and, to a lesser extent, in RPMΦs when compared with controls (Figure 2F). When considered in the context of gene expression and enzyme activity assays, these results support a model in which activated Drosophila macrophages undergo a dramatic metabolic remodeling towards AG during bacterial infection.

**Hif1α and Ldh activities are increased in Drosophila macrophages during acute phase of infection**

Since Hif1α can induce AG in both murine and Drosophila cells [24] [36] [37], we examined the possibility that this transcription factor also promotes glucose catabolism within activated macrophages. Although Hif1α is known to be continuously expressed in almost all tissues and regulated predominantly at the post-translational level, we observed that Hif1α mRNA was significantly elevated in APMΦs (Figure 3D). To determine if this increase correlates with elevated expression of Hif1α target genes, we used a transgenic β-galactosidase reporter under the control of a HRE (HRE-LacZ), which is primarily induced by HIF1α [46], however the involvement of other transcription factors can not be entirely excluded. Although some cells exhibited HRE-LacZ expression in uninfected individuals, the number of β-galactosidase positive macrophages rose dramatically in flies during the acute phase of infection (Figure 3A). These results suggest that Hif1α activity is increased in APMΦs and confirms the previously reported expression pattern of glycolytic genes (see Figure 2 - figure supplement 1 A-F).
As increased lactate production is a hallmark of AG, we examined *Ldh* expression in macrophages using a transgene that expresses a Ldh-mCherry fusion protein from an endogenous *Ldh* promoter. Expression of Ldh-mCherry in adult flies harboring the *HmlGal4>UAS-eGFP* reporter revealed that macrophages from uninfected adults expressed *Ldh* at levels that markedly exceeded expression in other tissues, perhaps indicating that these cells are primed to generate lactate prior to infection (Figure 3B), since the Ldh-mCherry pattern did not change significantly after infection (data not shown). *Ldh* expression, however, was significantly up-regulated in APMФs (Figure 3C), further supporting our observation that *S. pneumoniae* induces Ldh activity (Figure 2E), which is in agreement with elevated NADH levels (Figure 2G). The regulation of the *Ldh* expression by Hif1α in activated immune cells was verified by knocking down Hif1α expression in macrophages 24 hours before infection (*Hml>Hif1α[RNAi]*)). This strategy not only reduced Hif1α expression within APMФs (Figure 4 – figure supplement 1 G), but also led to the loss of the ability to increase *Ldh* expression in APMФs, indicating the Hif1α is essential for the elevated Ldh activity in APMФs (Figure 3E).

**Hif1α promotes aerobic glycolysis in *Drosophila* macrophages during bacterial infection**

To determine if the observed increase in Hif1α activity is necessary to trigger AG in stimulated macrophages, we used *Hml>Hif1α[RNAi]* and examined the metabolic consequences. This treatment led to abrogation of the metabolic changes associated with AG. Following infection, APMФs expressing *Hif1α[RNAi]* did not accumulate NBDG (Figure 4A), and failed to increase expression of glycolytic genes (with the exception of Gpdh1) (Figure 4B). Moreover, these *Hml>Hif1α[RNAi]* expressing cells exhibited no increase in either Pgi or Ldh enzyme activity and displayed decreased NADH levels when compared with controls (Figure 4D, E, F). These results indicate that Hif1α activity is essential for inducing AG in macrophages during the immune response.
As a complement to these cell-specific studies of Hif1α, we also used Hml-Gal4 driving UAS-Ldh[RNAi] (Hml>Ldh[RNAi]) to reduce Ldh expression within APMΦs. Intriguingly, while this approach successfully reduced Ldh activity in macrophages (Figure 4G), the metabolic consequences were relatively mild. Within APMΦs, Hml>Ldh[RNAi] did not disrupt NBDG uptake and Pgi activity remained elevated (Figure 4C and 4I). Twenty-four hours after infection, however, we observed that NADH in Hml>Ldh[RNAi] macrophages failed to increase to the levels observed in infected controls (Figure 4H), thus revealing that increased Ldh activity is required for full metabolic reprogramming of Drosophila macrophages in response to bacterial infection.

Hif1α-mediated aerobic glycolysis in APMΦs causes systemic metabolic changes

As we have shown previously [41], the systemic metabolic adaptation of carbohydrate metabolism is intimately linked to the effective function of the immune system during the streptococcal infection. Therefore, we focused on the characterization of systemic carbohydrate metabolism during the acute phase of infection in Hml>Hif1α[RNAi] and Hml>Ldh[RNAi] flies (Figure 5). Both control genotypes underwent the expected metabolic response during the acute phase of streptococcal infection. A significantly raised level of circulating glucose was accompanied by a strong depletion of glycogen stores in tissues. While the Hif1α silencing completely suppressed the infection-induced changes in carbohydrate metabolism, infected Hml>Ldh[RNAi] flies still significantly increased circulating glucose, albeit to a lesser extent than the infected controls (Figure 5A). Although the glycogen stores appeared to be lowered in Hml>Ldh[RNAi] flies upon infection, the decrease was statistically insignificant (Figure 5B). Imporantly, the macrophage-specific knockdown of either Hif1α or Ldh suppressed the occurrence of infection-induced increase in circulating lactate titer (Figure 5C). These results show that APMΦs are prominent lactate producers during the acute phase of the infection, and
suggest that only full activation of APMs with Hif1α-induced metabolic changes leads to reprogramming of systemic carbohydrate metabolism.

Our results suggest that Drosophila macrophages activate AG and systemic metabolic changes in order to mount a successful immune response. In support of this hypothesis, we observed a significant decrease in the viability of adult flies expressing either Hml>Hif1α[RNAi] or Hml>Ldh[RNAi] following S. pneumoniae infection. By 72 hours post infection, 25% of Hml>Hif1α[RNAi] flies died compared to 7% of controls and the medium time to death (MTD) in Hml>Hif1α[RNAi] flies was 10 days compared to 23 days in controls (Figure 6A). Moreover, pathogen load in Hml>Hif1α[RNAi] flies was substantially elevated when compared with controls at the second and third day post-infection (Figure 6C). We observed similar effects in Hml>Ldh[RNAi] flies, which upon S. pneumoniae infection exhibited a decreased survival rate, a MTD of 9 days relative to the 18 days observed in controls, and elevated bacterial load during days 2 and 3 post-infection (Figure 6B and 6D). These results reveal that Hif1α and Ldh serve essential roles in both surviving infection and bacterial killing and demonstrate how shift towards AG associated with systemic metabolic changes in activated macrophages is required to mount a successful immune response.

Discussion
Mammalian macrophages stimulated by bacteria have been shown to temporarily rewire their metabolism towards AG to develop an adequate bactericidal response [45], [47], [48]. Although well established in mammals, such metabolic adaptation has not been experimentally tested in insect macrophages to date. We show here that Drosophila macrophages activated by bacterial infection undergo a dramatic remodeling of cellular metabolism. We demonstrate that acute phase macrophages exhibit hallmarks of AG, such as elevated uptake of glucose, increased expression and activity of glycolytic genes, elevation of NADH, and preferential LDH-mediated conversion of pyruvate to lactate. Through macrophage-specific gene knockdown, we identified Hif1α to be essential for the induction of increased glycolytic flux as well as for the increased activity of LDH. Both Hif1α and Ldh are necessary for the full development of infection-induced changes of systemic carbohydrate metabolism and for the resistance to bacterial infection.

A major takeaway of our work is that the cellular response to bacterial infection is an energetically challenging process that imposes significant metabolic demands upon the host. Our findings demonstrate that Drosophila macrophages meet the metabolic demands by inducing AG during the acute phase of S. pneumoniae infection, as evident by increased expression of glycolytic enzyme genes and elevated NADH levels. This increase in LDH enzyme activity in the absence of elevated of TCA cycle activity suggests that macrophages preferentially convert pyruvate to lactate and is consistent with the elevated concentration of lactate observed in hemolymph. However, we find that this metabolic adaptation is temporary, as AG is terminated during the resolution phase of infection. This latter observation is important because it reveals that macrophages temporally regulate metabolic flux throughout an infection and establishes Drosophila as a powerful model for exploring the molecular mechanisms that control immune cell metabolism.

Our findings also extend the similarities between fly and mammalian models of macrophage polarization, as we identified Hif1α and Ldh to be crucial for the establishment and maintenance of AG
in acute phase macrophages. The importance of these factors is demonstrated by the macrophage-
specific Hif1α knockdown experiment described above, which abolished many of the hallmark
characteristics of AG, including expression of the Ldh gene. This finding highlights the conserved and
ancient role for Hif1α in regulating the switch between glycolytic and oxidative metabolism [49], and
suggests that this function evolved as a means of allowing cells to quickly adapt to changing
physiological conditions and cell-specific metabolic needs. The role of Hif1α in regulating this switch is of
significant interest because while this transcription factor is classically associated with the response to
hypoxia, our study adds to the growing list of examples in which Hif1α remodels cellular metabolism in
the context of cell proliferation, activation, and competition, even under normoxic conditions [50].
Moreover, our finding is particularly intriguing in light of the fact Hif1α also serves a key role in
promoting AG in neoplastic tumors cells [36] [37] [51]. Therefore, our studies of fly macrophages
provide an new in vivo system to understand how Hif1α promotes cell activity by modulating central
carbon metabolism.

While Hif1α drives AG in Drosophila macrophages via transcriptional regulation of target genes,
the role of Ldh in these cells is more complicated. Although acute phase macrophages still consume
more glucose upon Ldh knockdown, these cells exhibit significantly lower Pgi activity and NADH levels
and the titer of circulating lactate also drops. Our results suggest that even though Ldh acts only at the
last step of AG, its role is essential for full metabolic reprograming and efficient function of immune
cells. Drosophila Ldh is, similarly to its mammalian ortholog, responsible for the reduction of pyruvate to
lactate, linked with the regeneration of NAD⁺ from NADH. However, this single reaction has an immense
impact on cellular metabolism. Both accumulation of pyruvate and lack of NAD⁺ can become limiting in
cells with high glycolytic flux [45]. In addition, Ldh-dependent removal of cytosolic pyruvate was recently
found to be essential to prevent pyruvate entry to mitochondria and subsequent change of TCA cycle
course [37], [51].
Although not targeted in our study, changes in mitochondrial metabolism are also closely associated with AG and should be the focus of future studies of activated Drosophila macrophages. The interconnection between the transcriptional activity of Hif1α and the change of mitochondrial metabolism in Drosophila was recently elucidated, while several direct targets of its transcriptional activity leads to an inhibition of the classical course of TCA cycle [52] [53]. One of the well understood mechanisms is prevention of pyruvate entry to TCA cycle is caused by increased kinase activity of Pyruvate dehydrogenase kinase1 (Pdk1). PDK1-mediated phosphorylation of Pyruvate dehydrogenase (PDH) inhibits directly its enzymatic function that is essential for pyruvate conversion to acetyl-CoA [52]. This event causes a cytoplasmic accumulation of TCA cycle intermediates and thus promotes a secondary wave of Hif1α stabilization through inhibition of Prolyl hydroxylase dehydrogenase (PHD) under normoxic conditions [54] [55]. The change of TCA cycle is further needed for mitochondrial production of ROS that are transferred to the phagolysosome for bacterial killing [56] [57].

We further demonstrate that both Hif1α and Ldh are crucial not only for the full macrophage activation, but also for bactericidal function of the immune cells, with the rearrangement of macrophage metabolism towards AG being essential for resistance to infection and host survival. An important aspect of AG is the functional dependence of macrophages on sufficient supply of external energy resources, as demonstrated in both mammalian and insect phagocytes [41], [58], [59], [18], [60] and documented here by increased consumption of glucose. Immune cells therefore generate systemic factors to secure sufficient supply of nutrients by altering the function of other organs and by regulating systemic metabolism [61], [41]. Even though the identification of concrete signaling factors is out of scope of this work, there are several candidate molecules in Drosophila, known to be produced by activated macrophages as a reaction to the metabolic state of the cell. Although it is likely that multiple factors will be involved in this process, we can presume that these factors will be reflecting metabolic state of the cells (e.g., extracellular adenosine), or linked to the transcriptional program causing the
switch towards AG (e.g. Imaginal morphogenesis protein late 2 (ImpL2)). In our previous work, we have shown that the systemic metabolic switch upon infection is dependent on extracellular adenosine, which is produced by the activated immune cells [41] [61]. The production of adenosine directly reflects a metabolic state of the cell such as increased consumption of ATP [62], or accelerated occurrence of methylation events [63] [64]. Expression of ImpL2 was shown to be regulated by Hif1α [65] and since ImpL2 was previously identified as a mediator of cancer-induced loss of energy reserves in flies due to its anti-insulin role [66] [67], it could represent another link between AG in macrophages and changes in systemic metabolism, ensuring sufficient supply of energy resources.

Finally, our findings raise an interesting question regarding the links between AG and the ability of immune cells to quickly respond to infection. Recent studies of mammalian macrophages metabolism revealed that AG is essential development of innate immune memory - called trained immunity [68]. The mechanism of trained immunity relies in chromatin remodeling by epigenetic factors that enable cells to react with higher efficiency in response to re-infection by a particular pathogen [69]. Since many of chromatin remodeling enzymes need cofactors (such as acetyl-CoA, NAD+, α-KG) for remodeling of epigenetic landscape, their function can thus be influenced by the metabolic state of the cell. Induction of AG leads to the accumulation of many cofactors that are essential for a proper function of these enzymes [69] [70]. The concept of trained immunity is valid not only for mammals, but is rather present in many invertebrate clades (where it is called immune priming [71] [72]. Our observation of AG as a characteristic feature of activated Drosophila macrophages thus raises a question of its importance also for the development of trained immunity in insects and other invertebrates. Taken together, our findings demonstrate how the molecular mechanisms that control AG induction in Drosophila macrophages exhibit a surprisingly high level of evolutionary conservation between mammals and insect, thus emphasizing that this metabolic switch is essential for survival of infection and hinting at the potential role for AG in the development of immune memory.
In conclusion, we have shown that infection-induced systemic changes of carbohydrate metabolism are associated with changes of macrophage cellular metabolism, and both can be affected by macrophage-specific Hif1α and Ldh knockdown. Our data thus link the metabolic state of macrophages with the systemic metabolic changes. Based on our previous research on the selfish nature of the immune system under challenge [22], we envision that the shift in the cellular metabolism of macrophages leads to the production of signals that alter the systemic metabolism to secure sufficient energy supply necessary for the macrophages to fight the infection. By linking the induction of macrophage polarization with systemic metabolism and systemic outcomes in vivo, our experimental system can aid future research towards better understanding of the immune system and of diseases related to its malfunction.

Acknowledgements

The authors acknowledge funding by Grant Agency of the Czech Republic to TD (Project 17-16406S; www.gacr.cz). J.M.T. and G.C. were supported by R35 MIRA 1R35GM119557 from NIGMS/NIH. S. pneumoniae bacterial strain was obtained from David Schneider. We thank to Pablo Wappner and Bruno Lemaitre, who kindly provided us with HRE-HRE-CRE-LacZ and HmlΔ-Gal4 UAS-eGFP transgenic fly lines. Other fly stocks were obtained from the Bloomington Center (Bloomington, IN) and the VDRC (Vienna, Austria). We thanks to reviewing editor prof. Utpal Banerjee and prof. Ulrich Theopold for interesting comments on our work and hints for discussion improvement.

Declaration of interests

The authors declare no competing interests.
References


http://g3journal.org/lookup/doi/10.1534/g3.112.002584.

https://www.ahajournals.org/doi/10.1161/CIRCRESAHA.117.311401.


http://dx.plos.org/10.1371/journal.pbio.1002135.


Figure 1. Graphical representation of experimental approach

(A) The natural progress of streptococcal infection with highlighted sampling times of acute and resolution phase of infection. The Y axis indicates percentage of surviving adults. (B) The approach to isolation of hemocytes, subsequently assayed for gene expression and enzymatic activities. Macrophages sorted from flies at the respective time points post-infection represent acute phase macrophages (APMΦs; 24 hpi) and resolution phase macrophages (RPMΦs; 120 hpi). Control flies were analyzed at the same time points after receiving injection of PBS. hpi, hours post-infection; FACS, fluorescence-activated cell sorting; S.p., Streptococcus pneumoniae.
**Figure 2. Streptococcal infection enhances glycolysis in acute phase macrophages**

(A-B) Fluorescent images of the dorsal view of the abdomens of infected and control (both Hml>GFP) flies at 24 and 120 hpi showing NBDG distribution among the tissues (A) and at a higher magnification (B) Images represent a minimum of ten observations of a similar pattern. (C) Scheme of glycolysis and TCA cycle highlighting significant changes in the quantified expression of the indicated genes at 24 and 120 hpi. The expression levels of the mRNA were measured relative to that of the ribosomal protein 49 (rp49) and the statistical significance (p<0.05) was tested using ANOVA (for data see Figure 2 – figure supplement 1). Up-regulated genes are shown in red, down-regulated in green; gray indicates no statistically significant difference. (D, E, F) Enzymatic activities of Phosphoglucone isomerase (Pgi) (D) and Lactate dehydrogenase (Ldh) (E), and level of NADH (F) at 24 and 120 hpi measured in the homogenate of hemocytes isolated from infected and control flies. The levels of enzymatic activities and NADH concentration were normalized per ten thousand of cells per sample. (G) The concentration of circulating lactate measured in the hemolymph of infected and control flies at 24 and 120 hpi. In all plots (D-G), individual dots represent biological replicates. Values are mean ± SD, asterisks mark statistically significant differences (*p<0.05; **p<0.01; ***p<0.001).

**Figure 3. Macrophage-specific activities of Hif1α and Ldh increase upon infection**

(A) X-gal staining of infected and control flies bearing the HRE-LacZ reporter construct. Images represent a minimum of ten observations of a similar pattern. (B) An uninfected Hml>GFP, Ldh-mCherry adult fly 24 hpi shows localization of the Ldh reporter activity (red) to many of the immune cells (green). The image is a Z-stack at maximal projection of 25 confocal slices. (C-D) Expression of Ldh (C) and Hif1α (D) mRNAs in hemocytes isolated from infected and control flies (both Hml>GFP) 24 and 120 hpi. (E) Expression of Ldh mRNA in hemocytes of infected and control Hml>GFP flies with and without a hemocyte-specific knockdown of Hif1α at 24 hpi. In all plots (C-E), expression levels, normalized against rp49, are given as fold change (F.C.) relative to levels in PBS-injected Hml>GFP controls 24 hpi, arbitrarily set to 1. Individual dots represent biological replicates. Values are mean ± SD, asterisks mark statistically significant differences (*p<0.05; **p<0.01; ***p<0.001).
Figure 4. Effects of Hif1α and Ldh hemocyte-specific knockdown on macrophage metabolism

(A) Dorsal view of the abdomens of S.p.-infected flies (24 hpi) showing distribution of the fluorescent NBDG probe. Controls (left) are compared to flies subjected to hemocyte-specific knockdown of Hif1α. Images represent a minimum of ten observations of a similar pattern. (B) Schematic representation of gene expression of metabolic enzymes in hemocytes of infected control flies (left) and flies with Hif1α hemocyte-specific knockdown (right) at 24 hpi. The expression levels of the mRNA were measured relative to that of rp49 and the statistical significance (p<0.05) was tested using ANOVA (for data see Figure 4 – figure supplement 1). Up-regulated genes are shown in red; gray indicates no statistically significant difference. (C) Dorsal view of the abdomens of S.p.-infected flies (24 hpi) showing distribution of the fluorescent NBDG probe. Controls (left) are compared to flies subjected to hemocyte-specific knockdown of Ldh. Images represent a minimum of ten observations of a similar pattern. (D-F) Enzymatic activity of Ldh (D), level of NADH (E), and enzymatic activity of Pgi (F) at 24 and 120 hpi measured in lysates of hemocytes isolated from infected and non-infected control flies and flies with Hif1α hemocyte-specific knockdown. (G-I) Enzymatic activity of Ldh (G), level of NADH (H), and enzymatic activity of Pgi (I) at 24 and 120 hpi measured in lysates of hemocytes isolated from infected and non-infected control flies and flies with Ldh hemocyte-specific knockdown. In all plots (D-I), the enzyme activities and NADH concentrations were normalized per ten thousand cells per sample. Individual dots represent biological replicates. Values are mean ± SD, asterisks mark statistically significant differences (*p<0.05; **p<0.01; ***p<0.001).

Figure 5. Systemic effects of Hif1α and Ldh hemocyte-specific knockdown

(A-C) The concentration of circulating glucose (A), glycogen stores (B) and circulating lactate (C) in infected and non-infected flies with Hif1α or Ldh hemocyte-specific knockdown and their respective controls at 24 hpi. The concentrations of metabolites were normalized to the amount of proteins in each sample. Individual dots in the plot represent biological replicates. Values are mean ± SD, asterisks mark statistically significant differences (*p<0.05; **p<0.01; ***p<0.001).
Figure 6. Effects of Hif1α and Ldh hemocyte-specific knockdown on resistance to infection

(A-B) The survival rate of infected flies of control genotype and flies with hemocyte-specific Hif1α (A) and Ldh (B) knockdown. Vertical dotted lines denote medium time to death for each genotype; survival rate during the first 120 h is shown in detail. Three independent experiments were performed and combined into one survival curve. The average number of individuals per replicate was more than 500 for each genotype. (C-D) Colony forming units (CFUs) obtained from infected flies of control genotype and flies with hemocyte-specific Hif1α (C) and Ldh (D) knockdown at 0, 24, 48, and 72 hpi. Individual dots in the plot represent the number of bacteria raised from one individual. The data show results merged from three independent biological replicates.

Figure 2 – figure supplement 1. Gene expression of glycolytic enzymes is increased in acute phase macrophages

Gene expression of glycolytic (HexA (A), Pgi (B), Pfk (C), Tpi (D), Gapdh1 (E), Eno (F)) and TCA (Cis (G), Scsα1 (H), CG10219 (I)) genes in hemocytes of infected and control flies (both Hml>GFP) at 24 and 120 hpi. The mRNA expression levels, normalized against rp49, are given as fold change (F.C.) relative to expression of noninfected controls. Individual dots represent biological replicates. Values are mean ± SD, asterisks mark statistically significant differences (*p<0.05; **p<0.01; ***p<0.001).

Figure 4 – figure supplement 1. Gene expression of glycolytic enzymes is not increased in acute phase macrophages with Hif1α knock-down

(A-F) Gene expression of glycolytic genes (HexA (A), Pgi (B), Pfk (C), Tpi (D), Gapdh1 (E), Eno (F)) in hemocytes of infected and control Hml>GFP flies and flies with hemocyte-specific Hif1α knockdown at 24 hpi. (G-H) Gene expression of Hif1α (G) and Ldh (H) at 24 hpi in infected and control Hml>GFP flies and flies with hemocyte-specific Hif1α knockdown representing the efficiency of RNAi treatment. The mRNA expression levels, normalized against rp49, are given as fold change (F.C.) relative to expression of noninfected controls. Individual dots represent...
biological replicates. Values are mean ± SD, asterisks mark statistically significant differences (*p<0.05; **p<0.01; ***p<0.001).

MATERIALS AND METHODS

<table>
<thead>
<tr>
<th>Reagent type (species) or resource</th>
<th>Designation</th>
<th>Source Reference</th>
<th>Identifier</th>
<th>Additional Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain, strain background (Streptococcus pneumoniae)</td>
<td>EJ1 strain</td>
<td>Provided by David Schneider</td>
<td></td>
<td>Dilution 20 000 units</td>
</tr>
<tr>
<td>Chemical compound, drug</td>
<td>TRIzol Reagent</td>
<td>Invitrogen</td>
<td>Cat# 15-596-018</td>
<td></td>
</tr>
<tr>
<td>Chemical compound, drug</td>
<td>Superscript III Reverse Transcriptase</td>
<td>Invitrogen</td>
<td>Cat# 18080044</td>
<td></td>
</tr>
<tr>
<td>Chemical compound, drug</td>
<td>2x SYBR Master Mix</td>
<td>Top-Bio</td>
<td>Cat# T607</td>
<td></td>
</tr>
<tr>
<td>Chemical compound, drug</td>
<td>2-NBDG</td>
<td>Thermo Fisher Scientific</td>
<td>Cat# N13195</td>
<td></td>
</tr>
<tr>
<td>Chemical compound, drug</td>
<td>X-gal</td>
<td>Sigma</td>
<td>Cat# B4252</td>
<td></td>
</tr>
<tr>
<td>Commercial assay, kit</td>
<td>Glucose (GO) Assay Kit</td>
<td>Sigma</td>
<td>Cat# GAGO20-1KT</td>
<td></td>
</tr>
<tr>
<td>Commercial assay, kit</td>
<td>Bicinchoninic Acid Assay Kit</td>
<td>Sigma</td>
<td>Cat# BCA1</td>
<td></td>
</tr>
<tr>
<td>Commercial assay, kit</td>
<td>Lactate Assay Kit</td>
<td>Sigma</td>
<td>Cat# MAK064</td>
<td></td>
</tr>
<tr>
<td>Commercial assay, kit</td>
<td>Lactate Dehydrogenase Activity Assay Kit</td>
<td>Sigma</td>
<td>Cat# MAK066</td>
<td></td>
</tr>
<tr>
<td>Commercial assay, kit</td>
<td>Phosphoglucose Isomerase Colorimetric Assay Kit</td>
<td>Sigma</td>
<td>Cat# MAK103</td>
<td></td>
</tr>
<tr>
<td>Genetic reagent (Drosophila melanogaster)</td>
<td>HmlG4G80: w*; HmlΔ-Gal4*; P(tubPGal80ts)*</td>
<td>Cross made in our laboratory by Tomas Dolezal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genetic reagent (Drosophila)</td>
<td>Hml&gt;GFP: w; HmlΔ-Gal4 UAS-eGFP</td>
<td>Provided by Bruno Lemaitre</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genetic reagent (Drosophila melanogaster)</td>
<td>Hiř1α[RNAi]: P(KK110834) VIE-260B</td>
<td>Vienna Drosophila Resource Center</td>
<td>VDRC: v106504</td>
<td>FBst0478328</td>
</tr>
<tr>
<td>-----------------------------------------</td>
<td>----------------------------------</td>
<td>---------------------------------</td>
<td>----------------</td>
<td>------------</td>
</tr>
<tr>
<td>Genetic reagent (Drosophila melanogaster)</td>
<td>TRIp control: y[1] v[1]; P[y+[t7.7=CaryP]] attP2</td>
<td>Bloomington Drosophila Stock Center</td>
<td>BDSC: 36303</td>
<td>FBst0036303</td>
</tr>
<tr>
<td>Genetic reagent (Drosophila melanogaster)</td>
<td>KK control: y, w[1118]; P(attP,y+[], w[3'])</td>
<td>Bloomington Drosophila Stock Center</td>
<td>BDSC: 60100</td>
<td>FBst0060100</td>
</tr>
<tr>
<td>Genetic reagent (Drosophila melanogaster)</td>
<td>HRE-LacZ: HRE-HRE-CRE-LacZ</td>
<td>Provided by Pablo Wappner (Lavista-Llanos et al., 2002)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genetic reagent (Drosophila melanogaster)</td>
<td>Ldh-mCherry</td>
<td>Provided by Jason Tennessen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genetic reagent (Drosophila melanogaster)</td>
<td>w: w1118</td>
<td>Genetic background based on CantonS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sequence – based reagent</td>
<td>Cis forward: 5’ TTCGATTGACTCCAGCCTGG3’</td>
<td>KRD</td>
<td>CG14740</td>
<td>FBgn0037988</td>
</tr>
<tr>
<td>Sequence – based reagent</td>
<td>Cis reverse: 5’ AGCCGGGAACCACCTGTCC3’</td>
<td>KRD</td>
<td>CG14740</td>
<td>FBgn0037988</td>
</tr>
<tr>
<td>Sequence – based reagent</td>
<td>Ldh forward: 5’ CAGAGAAGTGGAACGCTGG3’</td>
<td>KRD</td>
<td>CG10160</td>
<td>FBgn0001258</td>
</tr>
<tr>
<td>Sequence – based reagent</td>
<td>Ldh reverse: 5’ CATGTTCGCCCAAAACGAG3’</td>
<td>KRD</td>
<td>CG10160</td>
<td>FBgn0001258</td>
</tr>
<tr>
<td>Sequence – based reagent</td>
<td>Eno forward: 5’ CAACATCCAGTCCAACAGG3’</td>
<td>KRD</td>
<td>CG17654</td>
<td>FBgn0000579</td>
</tr>
<tr>
<td>Sequence – based reagent</td>
<td>Eno reverse: 5’ GTTCTTGAAGTCCAGATCGT3’</td>
<td>KRD</td>
<td>CG17654</td>
<td>FBgn0000579</td>
</tr>
<tr>
<td>Sequence – based reagent</td>
<td>Gapdh1 forward: 5’ TTG TGG ATC TTA CCG TCC GC3’</td>
<td>KRD</td>
<td>CG12055</td>
<td>FBgn0001091</td>
</tr>
<tr>
<td>Sequence – based reagent</td>
<td>Gapdh1 reverse: 5’ CTCGAACACAGACGAA TGGG3’</td>
<td>KRD</td>
<td>CG12055</td>
<td>FBgn0001091</td>
</tr>
<tr>
<td>Sequence – based reagent</td>
<td>HexA forward: 5’ ATATCGGGCATGTATAT GGG3’</td>
<td>KRD</td>
<td>CG3001</td>
<td>FBgn0001186</td>
</tr>
<tr>
<td>Sequence – based reagent</td>
<td>HexA reverse: 5’ CAATTTCCGCTCACATAC</td>
<td>KRD</td>
<td>CG3001</td>
<td>FBgn0001186</td>
</tr>
<tr>
<td>Sequence – based reagent</td>
<td>Forward: 5’AGCTCACATTCTCAACATCG3’</td>
<td>Reverse: 5’TTTGATCACCAGATCTCTGCCA3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------------------</td>
<td>---------------------------------</td>
<td>-----------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pfk</td>
<td>KRD</td>
<td>CG4001</td>
<td>FBgn0003071</td>
<td></td>
</tr>
<tr>
<td>Pgi</td>
<td>KRD</td>
<td>CG8251</td>
<td>FBgn0003074</td>
<td></td>
</tr>
<tr>
<td>Rp49</td>
<td>KRD</td>
<td>CG7939</td>
<td>FBgn0002626</td>
<td></td>
</tr>
<tr>
<td>Hi1α</td>
<td>KRD</td>
<td>CG45051</td>
<td>FBgn0266411</td>
<td></td>
</tr>
<tr>
<td>CG10219</td>
<td>KRD</td>
<td>CG1065</td>
<td>FBgn0004888</td>
<td></td>
</tr>
<tr>
<td>Tpi</td>
<td>KRD</td>
<td>CG2171</td>
<td>FBgn0086355</td>
<td></td>
</tr>
<tr>
<td>Microsoft Excel</td>
<td>Microsoft Excel</td>
<td><a href="https://www.microsoft.com/">https://www.microsoft.com/</a></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fiji</td>
<td>Fiji</td>
<td>ImageJ - <a href="https://fiji.sc">https://fiji.sc</a></td>
<td>RRID:SCR_002285</td>
<td></td>
</tr>
<tr>
<td>Olympus FluoView 1000</td>
<td>Olympus</td>
<td>-</td>
<td>Olympus</td>
<td>RRID:SCR_017</td>
</tr>
</tbody>
</table>
Drosophila melanogaster strains

Flies were raised on a diet containing cornmeal (80 g/l), agar (10 g/l), yeast (40 g/l), saccharose (50 g/l) and 10% methylparaben (16.7 mL/l) and were kept in a controlled humidity environment with natural 12h/12h light/dark periods at 25 °C, except for those used in temperature-controlled Gal80 experiments. Flies bearing Gal80 were transferred at 29 °C 24 hours prior to infection in order to degrade temperature sensitive Gal 80 protein. Prior to experiments, flies were kept in plastic vials on 0% glucose diet (cornmeal 53.5 g/l, agar 6.2 g/l, yeast 28.2 g/l and 10% methylparaben 16.7 mL/l) for 7 days and transferred into fresh vials every second day without CO2 in order to ensure good condition of the food. Infected flies were kept on 0% glucose diet in incubators at 29 °C due to the temperature sensitivity of S. pneumoniae. Drosophila Stock Centre in Bloomington provided TRiP control and Ldh[RNAi] flies. Hif1α[RNAi] and KK control flies were obtained from Vienna Drosophila Resource Center. Ldh-mCherry strain was kindly provided by Jason Tennessen, HRE-LacZ from Pablo Wappner and Hml>GFP from Bruno Lemaitre. w1118 strain has a genetic background based on CantonS.

Bacterial strain and fly injection

The Streptococcus pneumoniae strain EJ1 was stored at -80°C in Tryptic Soy Broth (TSB) media containing 16% glycerol. For the experiments, bacteria were streaked onto agar plates containing 3% TSB and 100 μg/mL streptomycin and subsequently incubated at 37°C + 5% CO2 overnight. Single colonies were inoculated into 3 mL of TSB liquid media with 100 μg/mL of streptomycin and 100 000 units of catalase and incubated at 37°C + 5% CO2 overnight. Bacterial density was measured after additional 4 hours so it reached an approximate 0.4 OD600. Final bacterial cultures were centrifuged and dissolved in phosphate buffered saline (PBS) so the final OD reached A = 2.4. S. pneumoniae culture was kept on ice prior to injection and during the injection itself. Seven-day-old males (survival experiments, qPCR assays, measurement of metabolites and enzymatic activity) or females (X-gal staining, NBG assay) were anaesthetized with CO2 and injected with 50 nL culture containing 20 000 bacteria of S. pneumoniae or 50 nL of mock buffer (PBS) into the ventrolateral side of the abdomen using Eppendorf Femtojet Microinjector.

Pathogen load measurement

Sixteen randomly chosen flies per genotype and treatment were anaesthetized with CO2 and individually homogenized in 200 µL PBS using a motorized plastic pestle. Serial dilutions were plated
onto TSB agar plates and incubated at 37 °C overnight. The number of colonies was counted at 0, 24, 48
and 72hpi. Collected data were compared using Tukey’s multiple comparisons test in Graphpad Prism
software. Sidak’s multiple comparison correction was performed.

Survival analysis

Injected flies were kept at 29° C in vials with approximately 30 individuals per vial and were transferred
onto a fresh food every other day. Dead flies were counted daily. At least three independent
experiments were performed and combined into one survival curve created in Graphpad Prism
software, whilst the individual experiments showed comparable results. Average number of individuals
was more than 500 per each genotype. Data were analyzed by Log-rank and Grehan-Breslow-Wilcoxon
(more weight to deaths at early time points) tests.

Isolation of hemocytes

GFP-labeled hemocytes were isolated from HmlΔ-Gal4 UAS-eGFP male flies using a fluorescent activated
cell sorting (FACS). Approximately 200 flies were anaesthetized with CO2, washed in PBS and
homogenized in 600 μL of PBS using a pestle. Homogenate was sieved through a nylon cell strainer (⌀ 40
μm). This strainer was then additionally washed with 200 μL of PBS, which was added to the
homogenate subsequently. Samples were centrifuged (3 min, 6 °C, 3500 RPM) and the supernatant was
washed in ice cold PBS after each centrifugation (3x). Prior to sorting, samples were transferred to
polystyrene FACS tubes using disposable bacterial filter (⌀ 50 μm, Sysmex) and sorted into 100 μL of
TRIzol Reagent (Invitrogen) using S3TM Cell Sorter (BioRad). Sorted cells were verified by fluorescent
microscopy and by DIC.

Gene expression

Sorted hemocytes were homogenized using DEPC-treated pestle and RNA was extracted by TRIzol
Reagent (Invitrogen) according to manufacturer’s protocol. Superscript III Reverse Transcriptase
(Invitrogen) and oligo(dT)20 primer was used for reverse transcription. Amounts of mRNA of particular
genes were quantified on a CFX 1000 Touch Real-Time Cycler (Bio-Rad) using the TP 2x SYBR Master Mix
(Top-Bio) in three technical replicates with the following conditions: initial denaturation for 3 min at
95°C, then amplification for 15 sec at 94°C, 30 sec at 54°C, 40 sec at 72°C for 40 cycles and melting curve
analysis at 65 – 85°C/step 0.5°C. Primer sequences are entered in Key Resources Table. qPCR data were
analyzed with double delta Ct analysis, expressions or particular genes were normalized to the
expression of Ribosomal protein 49 (Rp49) in the same sample. Relative values (fold change) to control
were compared and are shown in the graphs. Samples for gene expression analysis were collected from
three independent experiments. Data were compared with Tukey’s multiple comparisons test in
Graphpad Prism software. Sidak’s multiple comparison correction was performed.

Glucose uptake
HmlΔ-Gal4 UAS-eGFP adults were placed on a cornmeal diet with added 200 µL of 2-NBDG (excitation/emission maxima of ~ 465/540 nm, 5 mg/mL stock – used 10 000x diluted, Thermo-Fisher), which was soaked into the surface of food, immediately after infection (flies analyzed at 24 hpi) or 96 hpi (flies analyzed at 120 hpi). After one day, flies were prepared for microscopy (Olympus IX71). Flies for glucose uptake analysis were collected from three independent experiments.

Activation of hypoxia response element (HRE)

X-gal staining was performed on infected HRE-HRE-CRE-LacZ females. Flies were dipped in 75% EtOH for one second in order to make their cuticle non-hydrophobic and dissected in PBS. Fixation was performed with 2.5% glutaraldehyde/PBS on LabRoller rotator for 7 minutes at room temperature. Adults were then washed three times in PBS. Next two washings were performed with a PT solution (1 mL 10xPBS (Ambion), 100 µL 1M MgCl2 x 6H2O, 300 µL 10% Triton, 8 mL dH2O, 320 µL 0.1M K4[Fe(CN)6], 320 µL 0.1 M K3[Fe(CN)6]) for 10 minutes. Finally, PT solution with few grains of X-gal (Sigma) was added. Samples were placed in a thermoblock at 37 °C and occasionally mixed and the colorimetric reaction was monitored. Reaction was stopped with three PBS washings at the same time for all samples. Samples for HRE activation evaluation were collected from four independent experiments.

Concentration of metabolites

Five flies were homogenized in 200 µL of PBS and centrifuged (3 min, 4 °C, 8000 RPM) for glycogen measurement. For lactate and glucose measurement, hemolymph was isolated from 25 adult males by centrifugation (14 000 RPM, 5 min) through a silicagel filter into 50 µL PBS.Half from all samples was used for quantification of proteins. Samples for glucose, glycogen and lactate measurement were denatured at 75 °C for 10 minutes, while samples for protein quantification were stored in -80 °C.

Glucose was measured using a Glucose (GO) Assay (GAGO-20) Kit (Sigma) according to the manufacturer’s protocol. Colorimetric reaction was measured at 540 nm. For glycogen quantification, sample was mixed with amyloglucosidase (Sigma) and incubated at 37 °C for 30 minutes. Bicinchoninic Acid Assay (BCA) Kit (Sigma) was used for protein quantification according to the supplier’s protocol and the absorbance was measured at 595 nm. Lactate Assay Kit (Sigma) was used for lactate concentration quantification according to the manufacturer’s protocol. The absorbance was measured at 570 nm. Samples for metabolite concentration were collected from six independent experiments. Measured data were compared in Graphpad Prism using Tukey’s multiple comparisons test. Sidak’s multiple comparison correction was performed.

Enzymatic activity

Enzymatic activity of Lactate dehydrogenase and Phosphoglucone isomerase was measured using Lactate Dehydrogenase Activity Assay Kit (Sigma) or Phosphoglucone Isomerase Colorimetric Assay Kit (Sigma), respectively, according to the supplier’s protocol in 10 000 FACS-sorted hemocytes per each sample. Colorimetric reaction was measured at 450 nm. Samples for enzymatic activity detection were collected.
from six independent experiments. Measured values were compared in Graphpad Prism software using Tukey’s multiple comparisons test. Sidak’s multiple comparison correction was performed.

Genotypes of experimental models

Figure 1
(B) Hml>GFP refers to HmlΔ-Gal4 UAS-eGFP/HmlΔ-Gal4 UAS-eGFP; +/+  
Figure 2
(A-B, D-G) Hml>GFP refers to HmlΔ-Gal4 UAS-eGFP/HmlΔ-Gal4 UAS-eGFP; +/+  
Figure 3
(A) HRE-LacZ refers to HRE-HRE-CRE-LacZ/ HRE-HRE-CRE-LacZ; +/+  
(B) Hml>GFP Ldh-mCherry corresponds to HmlΔ-Gal4 UAS-eGFP, Ldh-mCherry/HmlΔ-Gal4 UAS-eGFP, Ldh-mCherry; +/+  
(C-E) Hml>GFP refers to HmlΔ-Gal4 UAS-eGFP/HmlΔ-Gal4 UAS-eGFP; +/+  
(E) Hml>GFP Hif1α[RNAi] corresponds to HmlΔ-Gal4 UAS-eGFP/UAS-Hif1α[RNAi]; +/+  
Figure 4
(A, D-F) Hml x KK control corresponds to HmlΔ-Gal4 UAS-eGFP/KK control; +/+; and Hml>Hif1α[RNAi] refers to HmlΔ-Gal4 UAS-eGFP/UAS-Hif1α[RNAi]; +/+  
(B) Hml>GFP refers to HmlΔ-Gal4 UAS-eGFP/HmlΔ-Gal4 UAS-eGFP; +/+; and Hml>Hif1α[RNAi] refers to HmlΔ-Gal4 UAS-eGFP/UAS- Hif1α[RNAi]; +/+  
(C, G-I) Hml x TRiP control corresponds to HmlΔ-Gal4 UAS-eGFP/+; TRiP control/+; and Hml>Ldh[RNAi] refers to HmlΔ-Gal4 UAS-eGFP/+; UAS-Ldh[RNAi]/ +  
Figure 5
(A, B, C) Hml x TRiP control corresponds to HmlΔ-Gal4 UAS-eGFP/+; TRiP control/+; and Hml>Ldh[RNAi] corresponds to HmlΔ-Gal4 UAS-eGFP/+; UAS-Ldh[RNAi]/ +; and Hml x KK control corresponds to HmlΔ-Gal4 UAS-eGFP/KK control; +/+  
Figure 6
(A, C) Hml>Hif1α[RNAi] refers to HmlΔ-Gal4/+; P{tubPGal80ts}/UAS-Hif1α[RNAi]; and Hml x KK control corresponds to HmlΔ-Gal4/KK control; P{tubPGal80ts}/+  
(B, D) w x Ldh[RNAi] refers to +/+; UAS-Ldh [RNAi]/+; and Hml>Ldh[RNAi] corresponds to HmlΔ-Gal4/+; P{tubPGal80ts}/UAS-Ldh[RNAi]
**A**  
Images showing control and infected samples at 24 and 120 hpi.  
**B**  
Images showing Hml>GFP, NBDG, and the merge at 24 hpi.  
**C**  
Pathways and reactions at 24 hpi:  
glucose → HexA → glucose-6-phosphate → Pgi → fructose-6-phosphate → Pfk → fructose-1,6-bisphosphate → dihydroxyacetone phosphate → glyceraldehyde-3-phosphate → 1,3-biphosphoglycerate → Gapdh1 → 3-phosphoglycerate → 2-phosphoglycerate → Eno → phosphoenolpyruvate → pyruvate.  
**D**  
Graph showing Pgi activity at 24 and 120 hpi.  
**E**  
Graph showing Ldh activity at 24 and 120 hpi.  
**F**  
Graph showing NADH basal level at 24 and 120 hpi.  
**G**  
Graph showing circulating lactate at 24 and 120 hpi.
A. 24 hpi - ACUTE PHASE OF INFECTION
   120 hpi - RESOLUTION PHASE OF INFECTION

B. Hml>GFP; Ldh-mCherry
   500 μm

C. Expression of Ldh
   24 hpi, 120 hpi
   **p < 0.001
   Hml>GFP PBS, Hml>GFP S.p.

D. Expression of Hif1α
   24 hpi, 120 hpi
   **p < 0.001
   Hml>GFP PBS, Hml>GFP S.p.

E. Expression of Ldh
   Hml>GFP, Hif1α[RNAi]
   PBS injected, S.p. infected