Endocytic pathway mediates refractoriness of insect *Bactrocera dorsalis* to RNA interference

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RNA interference (RNAi) is a powerful and convenient tool for sequence-specific gene silencing, and it is triggered by double-stranded RNA (dsRNA). RNAi can be easily achieved in many eukaryotes by either injecting or feeding dsRNAs. This mechanism has demonstrated its potential in fundamental research on genetics, medicine and agriculture. However, the possibility that insects might develop refractoriness to RNAi remains unexplored. In this study, we report that the oriental fruit fly, *Bactrocera dorsalis*, became refractory to RNAi using orally administered dsRNA targeting endogenous genes. Furthermore, refractoriness to RNAi is not gene-specific, and its duration depends on the dsRNA concentration. RNAi blockage requires the endocytic pathway. Fluorescence microscopy indicated that in RNAi refractory flies, dsRNA uptake is blocked. Genes involved in the entry of dsRNAs into cells, including *chc*, *cog3*, *light* and others, are down-regulated in RNAi refractory flies. Increasing the endocytic capacity by improving F-actin polymerization disrupts RNAi refractoriness after both primary and secondary dsRNA exposures. Our results demonstrate that an insect can become refractory to RNAi by preventing the entry of dsRNA into its cells.

RNAi is a conserved mechanism by which endogenous genes are silenced by dsRNAs in a sequence-specific manner. dsRNAs can be delivered into animals by various methods, including injection, feeding and transgenic expression. These dsRNAs are then processed by a member of the RNase III family, Dicer, into siRNAs of approximately 21 nucleotides in length. The siRNA works as a guide and is loaded into the RNA-induced silencing complex (RISC), leading to sequence-specific mRNA cleavage. RNAi can be cell-autonomous or non-cell-autonomous. Cell-autonomous RNAi refers to RNAi that occurs within a single cell. Non-cell-autonomous RNAi refers to the ability of dsRNA to trigger RNAi in cells that are distant from the initial site of RNAi or the location where the dsRNA was introduced. For the efficient use of RNAi in pest control, the focus must be on non-cell-autonomous RNAi caused by the feeding of dsRNAs. The use of RNAi in pest management requires the dsRNA to be ingested in the lumen without being degraded, then taken up in the intestinal cells. Then, the dsRNA molecules can pass through the intestinal cells and into the body cavity, where they can act on other tissues, such as muscles.

Because RNAi is easy to induce and highly efficient, it has been widely used throughout the scientific field. It has become a basic method in functional genetic studies. Genome-wide screens for genes involved in many biological pathways have been successfully carried out using RNAi-based methods. Although RNAi-based therapy has not been fully realised, several achievements in related areas still provide hope for success. RNAi is also a promising tool in agricultural science, and especially in pest management, as an environmentally friendly pesticide.

RNAi experiments have been carried out in various insect orders, including the Diptera, Coleoptera, Hemiptera and Isoptera. Two landmark articles demonstrated the feasibility of the oral administration of dsRNA in insects, supporting the use of RNAi in insect pest control. However, RNAi in insects has yielded varying results. Among the insects in which RNAi has been investigated, some appear to be RNAi-insensitive. For example, feeding dsRNAs to adults of the Dipteran species *Drosophila melanogaster* failed to elicit RNAi. In the Lepidopteran species *Spodoptera litura*, gene silencing using dsRNA was observed only with injection; the feeding method failed. An attempt to silence the *nitroporin* 2 gene in 4th instar larvae of *Rhodnius prolixus* failed despite the large quantity of dsRNA that was used (80 µg).
dsRNA and the control

the mean of three independent biological replicates. Normalised target gene expression is reported relative to the expression after

second exposure to the type I interferon (IFN) system in mammalian cells. Unlike in that the insect can become refractory to dsRNA-induced RNAi. Here, we report that the insect B. dorsalis can become refractory to RNAi triggered by feeding. Refractoriness is caused by a decrease in the endocytic entry of dsRNA into the intestinal cells, thus preventing RNAi. It has been demonstrated that both dsRNAs and siRNAs activate the type I interferon (IFN) system in mammalian cells. Unlike in vertebrates, it is generally believed that IFN responses do not exist in invertebrates because the critical genes or major effectors of the IFN pathway are absent in these species. Recent studies have shown that the RNAi pathway plays an important role in invertebrate viral immunity. Robalino et al. (2004) injected dsRNAs derived from vertebrate immunoglobulin genes, fish non-coding genomic DNA, bacterial vector sequences, and the Taura syndrome virus into marine shrimp. Each of these sequences induced protection against infection with the white spot syndrome virus (WSSV). Administration of dsRNA that targets either virus-specific or non-specific sequences can trigger an antiviral response that controls viral infections in honey bees. However, importantly, this viral immunity was not sequence-specific; it could be activated by dsRNAs derived from any sequence.

To date, there is little, if any, evidence indicating that an organism can become refractory to dsRNA-induced RNAi. Here, we report that the insect B. dorsalis can become refractory to RNAi triggered by feeding. Refractoriness is caused by a decrease in the endocytic entry of dsRNA into the intestinal cells, thus preventing RNAi. Digital gene expression (DGE) and qPCR analysis show that several genes involved in endocytosis are down-regulated. Increasing the endocytic capacity by promoting actin assembly can reverse the refractoriness. The mechanism of RNAi refractoriness uncovered here might explain why RNAi is difficult to achieve in certain insects.

**Results and Discussion**

Feeding of dsRNAs targeting endogenous genes induces protection against secondary RNAi. Unlike vertebrates, invertebrates lack acquired immunity. However, growing empirical evidence suggests that previous exposure to a parasite can lead to increased protection in response to a subsequent challenge. In vertebrates, this phenomenon is termed “immune priming”. In this study, we aimed to determine if primary RNAi could further influence secondary RNAi in B. dorsalis. First, we tested the effects of prior RNAi using a dsRNA sequence targeting a non-endogenous gene on a subsequent RNAi by exposure of dsRNA targeting an endogenous gene. These dsRNAs were derived from enhanced green fluorescent protein (egfp) (186 bp), Discosoma sp. red fluorescent protein (dsred) (192 bp), the hly gene from Listeria monocytogenes (hly) (207 bp). The flies were divided into two groups. The challenged group (Ch) was fed an artificial diet containing one of the above dsRNAs for 6 hr. The other group, referred to as the naive group (Nv), was fed a normal artificial diet. Five days post-first exposure (dpe), both the challenged group and the naive group were orally administered a dsRNA targeting either the ribosomal protein L19 gene (205 bp).

The results showed that exposure to 92 bp and 494 bp egfp dsRNA on a second exposure to rpl19 dsRNA. The effect of 92 bp and 494 bp egfp dsRNA on a second exposure to spr dsRNA. The effect of 92 bp and 494 bp egfp dsRNA on a second exposure to spr dsRNA. Normalised target gene expression is reported relative to the expression after egfp dsRNA treatment, which was set to 1. All error bars represent the SE of the mean of three independent biological replicates. * indicates a statistically significant difference in spr or rpl19 expression between rpl19 dsRNA or spr dsRNA and the control egfp dsRNA treatments (P < 0.01).

![Figure 1](https://example.com/figure1.png)

Figure 1 | Feeding dsRNA that targets exogenous genes do not affect subsequent RNAi. (A) The effect of dsRNA that targets exogenous genes on a second exposure to rpl19 dsRNA. (B) The effect of dsRNA that targets exogenous genes on a second exposure to spr dsRNA. (C) The effect of 92 bp and 494 bp egfp dsRNA on a second exposure to rpl19 dsRNA. (D) The effect of 92 bp and 494 bp egfp dsRNA on a second exposure to spr dsRNA.
did not change the effect of a secondary RNAi targeting either rpl19 or spr. Both the naïve and the challenged group exhibited strong RNAi (Figure 1C, D). It indicates that the length of dsRNAs targeting exogenous genes do not affect the outcome.

Next, we used rpl19 dsRNA as the first exposure for the challenged group, whereas egfp dsRNA was used for the naïve group. rpl19 expression decreased by 65.2% after the first exposure (Figure S3A). The RNAi effect disappeared four days after the first RNAi (Figure S3A). As expected, the naïve group showed efficient RNAi after secondary exposure to a dsRNA targeting rpl19; in this group, rpl19 expression decreased by 66% at 5 dpe (Figure 2A). However, in the challenged group, after secondary RNAi, depletion of rpl19 could not be observed at 5 dpe. This phenomenon suggests that an initial exposure to rpl19 dsRNA prevents RNAi-induced gene silencing after a second exposure to the same dsRNA.

We next examined the duration of RNAi refractoriness. We set the time lag between the two RNAi exposures to 10, 20 or 30 days (Figure S4). First we examined rpl19 and spr gene expression in the untreated flies. Our results suggest that there is no expression level fluctuation for ion in the untreated flies. Our results suggest that there is no significant difference between the naïve group and the challenged group when secondary spr gene silencing was assessed at 20 dpe (Figure 2B). We then tested if a dsRNA targeting a gene other than rpl19 could elicit RNAi refractoriness. To address this question, we chose spr as a target gene for initial dsRNA exposure. The results showed that, in the challenged group, the initial exposure to spr dsRNA induced refractoriness towards to secondary exposure to both spr dsRNA and rpl19 dsRNA (Figure 2C, D). The refractory state primed by spr dsRNA protected B. dorsalis for 5 dpe after secondary exposure to both spr and rpl19 dsRNA. However, this refractory period was much shorter than that provoked by rpl19 dsRNA. There was no significant difference in gene expression between the challenged and the naïve groups fed either rpl19 dsRNA or spr dsRNA after 10 dpe (Figure 2C, D). These results indicate that a refractory state can be provoked by dsRNAs with different sequences. Because we did not observe this refractory state using egfp or other dsRNAs targeting exogenous genes, we assume that only dsRNAs targeting endogenous genes can trigger RNAi refractoriness.

**RNAi refractoriness is not sequence-specific and is influenced by the concentration of the priming dsRNA.** We next examined if RNAi refractoriness primed by rpl19 dsRNA was also effective against secondary RNAi targeting spr at 5 dpe. The results showed that prior ingestion of rpl19 dsRNA provided protection against subsequent RNAi targeting spr. Feeding the naïve group with spr dsRNA lead to an approximately 70% down-regulation of spr expression after secondary RNAi (Figure 2B). By contrast, the group initially challenged with rpl19 dsRNA showed no reduction in spr gene expression after secondary spr RNAi at 5 dpe (Figure 2B). There was no significant difference between the naïve group and the challenged group when secondary spr gene silencing was assessed at 20 dpe (Figure 2B). We then tested if a dsRNA targeting a gene other than rpl19 could elicit RNAi refractoriness. To address this question, we chose spr as a target gene for initial dsRNA exposure. The results showed that, in the challenged group, the initial exposure to spr dsRNA induced refractoriness towards to secondary exposure to both spr dsRNA and rpl19 dsRNA (Figure 2C, D). The refractory state primed by spr dsRNA protected B. dorsalis for 5 dpe after secondary exposure to both spr and rpl19 dsRNA. However, this refractory period was much shorter than that provoked by rpl19 dsRNA. There was no significant difference in gene expression between the challenged and the naïve groups fed either rpl19 dsRNA or spr dsRNA after 10 dpe (Figure 2C, D). These results indicate that a refractory state can be provoked by dsRNAs with different sequences. Because we did not observe this refractory state using egfp or other dsRNAs targeting exogenous genes, we assume that only dsRNAs targeting endogenous genes can trigger RNAi refractoriness.

![Figure 2](https://www.nature.com/scientificreports/) Feeding dsRNA that targets endogenous genes induces protection against subsequent RNAi. (A) RNAi refractoriness to secondary rpl19 dsRNA exposure, primed by rpl19 dsRNA. (B) RNAi refractoriness to secondary spr dsRNA exposure, primed by rpl19 dsRNA. (C) RNAi refractoriness to secondary spr dsRNA exposure, primed by spr dsRNA. (D) RNAi refractoriness to secondary rpl19 dsRNA exposure, primed by spr dsRNA. Normalised target gene expression is reported relative to the expression after egfp dsRNA treatment, which was set to 1. All error bars represent the SE of the mean of three independent biological replicates. * indicates a statistically significant difference in spr or rpl19 expression between rpl19 dsRNA or spr dsRNA and the control egfp dsRNA treatments (P < 0.01). Different letters indicate a significant difference in rpl19 or spr expression among the rpl19 dsRNA or spr dsRNA treatments (P < 0.01).
We next explored if the concentration of dsRNA used in the first exposure influenced RNAi refractoriness. The results showed that 100 ng/µl rpl19 dsRNA decreased rpl19 expression by 38%, but that 10 ng/µl rpl19 dsRNA did not decrease rpl19 expression (Figure 3A). Both dsRNA concentrations could block RNAi-mediated gene silencing after secondary exposure to two different dsRNAs 5 dpe. Nevertheless, the refractory periods were shorter than those induced by the 1000 ng/µl treatment. In the group treated with 100 ng/µl, secondary exposure 10 dpe decreased rpl19 expression by 40% in the challenged group and by 70% in the naive group (Figure 3B). No difference could be observed between the naive and challenged groups after secondary exposure 20 dpe (Figure 3B). This shortened period of refractoriness was more obvious when analysing its effect on a second exposure to spr dsRNA; in this case, the RNAi refractory period lasted no longer than 10 days (Figure 3C). The refractoriness induced by the 10 ng/µl rpl19 dsRNA were even shorter than those induced by 100 ng/µl rpl19 dsRNA (Figure 3D, E). These results clearly illustrated that the duration of RNAi refractoriness to dsRNAs is correlated with the dsRNA concentration used for priming. In addition, we show that effective target gene silencing after the first exposure was not a prerequisite for RNAi refractoriness. Since that only the dsRNAs targeting endogenous genes could induce the RNAi refractoriness, this process might involves the dsRNA and target mRNA interaction. In addition, the fact that target gene silencing is not necessary for the RNAi refractoriness demonstrated that low level of dsRNA and target mRNA interaction, although not enough to silence target genes, is enough to elicit RNAi refractoriness. Thus, the high level of this interaction caused by high concentration of dsRNA guarantees a prolonged refractoriness.

**Disruption of the endocytic pathway inhibits dsRNA entry.** We next investigated the molecular mechanisms underlying RNAi

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**Figure 3** | RNAi refractoriness primed by different concentrations of rpl19 dsRNA. (A) The RNAi effect induced by oral administration of different concentrations of rpl19 dsRNA. (B) RNAi refractoriness to secondary rpl19 dsRNA exposure, primed by 100 ng/µl rpl19 dsRNA. (C) RNAi refractoriness to secondary spr dsRNA exposure, primed by 100 ng/µl rpl19 dsRNA. (D) RNAi refractoriness to secondary rpl19 dsRNA exposure, primed by 10 ng/µl rpl19 dsRNA. (E) RNAi refractoriness to secondary spr dsRNA exposure, primed by 10 ng/µl rpl19 dsRNA. Normalised target gene expression is reported relative to expression in the egfp dsRNA control, which was set to 1. All error bars represent the SE of the mean of three independent biological replicates. * indicates a statistically significant difference in spr or rpl19 expression between rpl19 dsRNA or spr dsRNA and the control egfp dsRNA treatments (P < 0.01). Different letters indicate a significant difference in rpl19 or spr expression among the rpl19 dsRNA or spr dsRNA treatments (P < 0.01).
In our conditions, the midgut is expected to be involved in dsRNA uptake\textsuperscript{31}. Cy3-labelled dsRNAs were used to track dsRNA in the midgut cells of naïve and challenged insects. Fluorescence microscopy revealed differences between the rpl19 dsRNA-challenged group and the egfp dsRNA naïve group after secondary exposure to rpl19 dsRNA (Figure 4A). In the naïve group, dsRNA begins to enter the cell after 30 minutes of incubation, and after another 30 minutes, the dsRNAs accumulate in a spot near the nucleus. This indicates that egfp dsRNA treatment did not impair the ability of dsRNAs from the second exposure to enter the midgut cells. However, in the rpl19 dsRNA-challenged group, the dsRNAs failed to enter the midgut cells, as indicated by labelled dsRNAs accumulating outside the cells after both 30 and 60 min. These fluorescence microscopy observations demonstrate that the challenged group failed to respond to dsRNA feeding due to impaired dsRNA cellular uptake.

Two mechanisms of dsRNA uptake have been identified: one is mediated by the SID-1 transmembrane protein, and the other is mediated by endocytosis\textsuperscript{30}. However, there is no sid-1 gene in several insect genomes, including that of D. melanogaster\textsuperscript{3}. Two independent works have shown that D. melanogaster relies on receptor-mediated endocytosis to take up dsRNA\textsuperscript{31,32}. Bafilomycin A1 (Baf), a specific inhibitor of vacuolar proton ATPases, is often employed to demonstrate the requirement for low endosomal pH. Our results showed that pretreating the B. dorsalis midgut with baf blocked dsRNA entry into the midgut cells (Figure 4B). This strongly supports the notion that, in B. dorsalis, the entry of dsRNA into the cells requires endocytosis. We next examined the expression of genes reported to be responsible for the cellular entry of dsRNA in D. melanogaster\textsuperscript{3}. These genes, such as chc, rab7, light and saposin, influence several crucial steps of endocytosis, including vesicle formation and transport, intracellular transport and lipid metabolism. Saleh et al. have shown that, in cell culture assays, RNAi against each of these genes inhibits dsRNA entry\textsuperscript{34}. qPCR results showed that, in the rpl19 dsRNA-challenged group, most of these genes were repressed 24 hr after secondary exposure (Figure 4C). For example, chc, a key gene required for clathrin-mediated endocytosis that encodes the clathrin heavy chain protein, was down-regulated by more than 60%. A similar level of down-regulation was observed for the rab7, arf72a, light (a vacuolar protein sorting Vsp41 orthologue) and vacuolar H+ -ATPase (V-H-ATPase) genes, which encode components of the endocytic vesicle trafficking and protein sorting pathways. Other genes, including members of the Golgi complex (COG) family (e.g., ldICp, cog3 and bet3) were also down-regulated. nina c, which is required for actin polymerization and cytoskeletal organization, was also down-regulated after the second exposure. These results raised the hypothesis that RNAi refractoriness is caused by a decrease in endocytosis. In order to find if the RNAi refractoriness is systemic, we directly injected rpl19 dsRNA into the flies to see if it could bypass RNAi refractoriness. The result showed that, in the challenged group, direct injection of dsRNA could not reduce the expression of the target gene at 5 dpe. This result demonstrated that this refractoriness is systemic (Figure 4D).

Actin assembly has been shown to be an essential element of endocytosis\textsuperscript{35}. Hydrogen peroxide (H$_2$O$_2$) induces the formation of cellular F-actin in a dose-dependent manner\textsuperscript{36}. To test the role of endocytosis in RNAi refractoriness, we investigated the ability of H$_2$O$_2$ to disrupt RNAi refractoriness. Applying H$_2$O$_2$ during the secondary exposure disrupted RNAi refractoriness; a second exposure with 100 ng/μl rpl19 dsRNA and 5% H$_2$O$_2$ resulted in a 41.1% decrease in target gene expression compared with the egfp dsRNA treatment (Figure 5A). This is also supported by fluorescence microscopy; we found that dsRNA successfully entered the cells after co-incubation with H$_2$O$_2$ (Figure 5B). Because increasing the endocytic capacity with H$_2$O$_2$ led to a higher level of RNAi-mediated gene silencing, our results strongly suggest that refractoriness to RNAi is mediated by a decrease in endocytosis.

**DGE analysis reveals that RNAi refractoriness is intrinsic.** Our results showed that 10 ng/μl rpl19 dsRNA could not efficiently

![Figure 4](https://www.nature.com/scientificreports/images/4-4.png)
represent the SE of the mean of three independent biological replicates. The normalised expression of the target gene is given relative to its expression in the control. A total of 5,793,444 and 6,363,092 clean reads were left after removing reads with adaptors, reads containing poly N and low quality reads from raw data (Table S1). We mapped the sequences to the reference transcriptome dataset of B. dorsalis, which contains 48,876 unigenes (Figure S6). This analysis identified 190 genes whose expression varied in response to feeding dsRNA compared with egfp dsRNA treatment. The gene set comprised 26 up-regulated and 164 down-regulated genes. Gene Ontology (GO) enrichment analysis indicated that the genes were enriched for 5 biological processes (p < 0.001), including translation regulator activity, cell motility and transport (Table S2). KEGG analysis of the differentially expressed genes showed that they function in several processes (Figure S7). The up-regulated genes mainly function in metabolism and translation, whereas the down-regulated genes primarily function in metabolism, translation and transcription, folding, sorting and degradation and transport and catabolism.

Importantly, several genes that play a crucial role in endocytosis were found to be down-regulated in the 10 ng/µl rpl19 dsRNA-treated flies (Table 1). chc and hsc70, which encode factors that work with auxin to uncoat CCV from cargo, as well as saposin, were found to be down-regulated. In addition, the expression of three genes encoding different F-actin isoforms, actin 3, actin 4 and actin 5, was reduced. The dynamic polymerization of actin has a central role in clathrin-mediated endocytosis, which reshapes the plasma membrane. The expression of three kinases, hexo kinase 2, map2k1 and pgk1, which regulate endocytosis, was also repressed. However, we failed to identify genes that had been previously characterised as involved in insect immunity; these genes encode components of the Toll, Imd and Jak/STAT pathways. This is consistent with the findings of Flenniken and Andino, who used microarrays to identify genes involved in the dsRNA-mediated antiviral response in honey bees; they did not identify any classical immunity genes. These results, as well as ours, suggest that dsRNA-mediated antiviral defence may involve unique genes and signal transduction cascades. In addition, we did not find core genes in the RNAi machinery, like dicer 2 and argonaute 2, in the DGE analysis, indicating the RNAi refractoriness is not due to the different activity of the RNAi machinery. The RNAseq results imply that endocytosis-mediated RNAi refractoriness also occurs at the first dsRNA exposure. To validate this hypothesis, we co-fed flies with 5% H₂O₂ and 10 ng/µl dsRNA, a dsRNA concentration that does not reduce rpl19 expression. However, rpl19 expression still decreased 33.5% relative to the egfp dsRNA treatment (Figure 5C). This result demonstrates that H₂O₂, which influences endocytosis, can also influence RNAi-induced gene silencing. This observation reinforces our conclusion that variation in endocytic capacity can influence RNAi and that RNAi insensitivity is linked to a decrease in endocytic activity.

**Conclusions**

Taken together, our findings indicate that B. dorsalis possesses a mechanism to down-regulate dsRNA-mediated RNAi. We also demonstrated that primed RNAi refractoriness involves clathrin-mediated endocytosis. We hypothesize that our findings will extend to other insect species. In line with this assumption, other works have already shown that receptor-mediated endocytosis influenced dsRNA entry in D. melanogaster. Our conclusion is also supported by the work of Whyard, who showed that feeding dsRNA encapsulated by transfection reagents could induce RNAi in four different Drosophila species, whereas direct dsRNA feeding did not work. This suggests that a mechanism involved in dsRNA entry is impaired in Drosophila species. We speculate that this defect is linked to a reduction in endocytosis. A recent report focused on the early response of Drosophila S2 cells to viruses. Interestingly, this work showed that receptors such as Sr-CI, Eater and Tepl were significantly down-regulated after pathogenic virus treatments, and significant changes in phagocytic activity were observed. It is known that together, Sr-CI and Eater contribute to more than 90% of dsRNA uptake into S2 cells. Considering the evolutionary conservation and functional relevance of the dsRNA entry pathway in intact organisms, this
change in membrane transport capacity might be common in host-pathogen interactions.

RNAi is an important viral defence mechanism in insects. The mechanism that we uncovered might affect the viral defences of insects and could explain the variability observed between the various physiological states that influence endocytosis. It also suggests that infection by a virus could influence RNAi silencing against a second virus or against a second exposure to the first virus. Furthermore, our work also has important consequences for the use of RNAi in other insects; it could explain why RNAi is difficult to achieve in some insect species. It also provides a solution to this problem by showing that promoting endocytosis enhances RNAi. In summary, our findings provide a perspective into mechanisms that allow invertebrates to protect their genetic information from non-self dsRNAs.

### Methods

#### Fly rearing

*B. drosalis* flies were reared at the Institute of Urban and Horticultural Pests at Huazhong Agricultural University. Adult flies were maintained in cages at 28°C under a 12 hr light: 12 hr dark photoperiod and fed a artificial diet consisting of 2.5% yeast extract, 7.5% sugar, 2.5% honey, 0.4% agar and 87% H2O.

#### Plasmid construction

Total RNA was extracted from adult flies using RNAiso Plus reagent (TaKaRa, Japan). First strand cDNA was synthesized using a PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, Japan). PCR fragments from each gene were cloned into the SacI and HindIII sites of the L4440 plasmid. The egfp and dsred fragment were cloned from the PUbnsEGFP plasmid and the PUbnsRED plasmid.

#### dsRNA preparation and quantification

A single HT115 (DE3) colony was cultured overnight in LB at 37°C with shaking at 220 rpm. The culture was diluted 100-fold in 800 mL 2 × YT supplemented with 75 μg/mL ampicillin and 12.5 μg/mL tetracycline and cultured at 37°C to an OD600 of 0.5. Production of T7 polymerase was induced with 0.4 mM IPTG, and the bacteria were incubated with shaking for an additional 4 hr at 37°C. Total nucleic acids were extracted. The bacterial pellets were resuspended in 1 M ammonium acetate/10 mM EDTA, and an additional volume of 1 M ammonium acetate was added. Isopropanol was added to the sample at a ratio of 2:1 (v:v) and resuspended. The sample was then incubated for 20 min at −20°C overnight and centrifuged at 12,000 g for 30 min. The nucleic acid pellet was resuspended in TE. For dsRNA quantification, the nucleic acids were treated with RNase-free DNAse (Promega, USA) and RNase A solution (Promega, USA). The concentration was determined using a NanoDrop 1000 (Thermo, USA), and cDNA was purified using Ampure beads (Agencourt, USA). The purified cDNA was used to prepare a library using a TruSeq™ DNA sample Prep Kit-Set A (Illumina, USA), and PCR amplification was performed using a TruSeq PE Cluster Kit (Illumina, USA). cDNA was purified using Ampure beads (Agencourt, USA). The purified cDNA was used to prepare a library using a TruSeq™ DNA sample Prep Kit-Set A (Illumina, USA), and PCR amplification was performed using a TruSeq PE Cluster Kit (Illumina, USA). Finally, the products were sequenced on an Illumina HiSeqTM 12000 System (Illumina, USA) and 100 bp pair-end reads were generated. Clean reads were mapped to a *B. drosalis* transcriptome dataset. Expression values were calculated in units of RPKM. Statistical analysis to identify differentially expressed genes was performed using an MA plot-based method with a random sampling model in DESeq2.

##### Immunofluorescence microscopy

dsRNA was fluorescently labelled using a Silencer siRNA Labelling Kit with Cy5 (Ambion, USA). Labelling of dsRNA was verified by decreased electrophoretic mobility compared with unlabelled dsRNA on an agarose gel. *B. drosalis* midgut tissue from both the challenged and naive groups were incubated with labelled dsRNA. For bafilomycin A1 treatment, midgut tissue was first incubated with 0.2 μM Baf for 30 minutes. Cy3-labelled dsRNA was then added to the reaction. The tissue was fixed for 20 min in 4% formaldehyde. Actin was visualized with Acti-stain™ 488 fluorescent phallolidin (Cytoskeleton, Inc., USA) following the instruction manual. Nuclei were counterstained with DAPI. Images were captured on an Olympus IX71 microscope driven by cellSens Dimension.

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**Table 1** | List of representative differentially expressed genes found by DGE

| Function          | Gene            | Fold change | q-value  |
|-------------------|-----------------|-------------|----------|
| Endocytosis       | cdc42           | 0.32        | 9.99E-03 |
|                   | chc             | 0.47        | 5.60E-07 |
|                   | hsc70           | 0.32        | 1.29E-12 |
|                   | ehd1            | 0.62        | 1.20E-02 |
| Peroxisome        | saposin         | 0.42        | 1.82E-10 |
| Phagosome         | vha16-1         | -0.20       | 2.55E-02 |
|                   | actin 3         | 0.38        | 6.19E-03 |
|                   | actin 5         | 1.01        | 1.09E-16 |
|                   | actin 4         | 0.21        | 1.44E-02 |
| Insulin signalling | hexokinase2     | 0.40        | 2.28E-08 |
|                   | man2k1          | 0.41        | 1.10E-02 |
| Glycolysis        | pgk1            | 0.46        | 9.63E-06 |
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