Apoptosis-related genes control autophagy and influence DENV-2 infection in the mosquito vector, *Aedes aegypti*

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Abstract

The mosquito *Aedes aegypti* is the primary urban vector for dengue virus (DENV) worldwide. Insight into interactions occurring between host and pathogen is important in understanding what factors contribute to vector competence. However, many of the molecular mechanisms for vector competence remain unknown. Our previous global transcriptional analysis suggested that differential expression of apoptotic proteins is involved in determining refractoriness vs susceptibility to DENV-2 infection in *Ae. aegypti* females following a DENV-infected blood meal. To determine whether DENV-refractory *Ae. aegypti* showed more robust apoptosis upon infection, we compared numbers of apoptotic cells from midguts of refractory and susceptible strains and observed increased numbers of apoptotic cells in only the refractory strain upon DENV-2 infection. Thereafter, we manipulated apoptosis through dsRNA interference of the initiator caspase, *Aedronc*. Unexpectedly, ds*Aedronc*-treated females showed both decreased frequency of disseminated infection and decreased virus titer in infected individuals. Insect caspases have also previously been identified as regulators of the cellular recycling process known as autophagy. We observed activation of autophagy in midgut and fat body tissues following a blood meal, as well as programmed activation of several apoptosis-related genes, including the effector caspase, *Casps7*. To determine whether autophagy was affected by caspase knockdown, we silenced *Aedronc* and *Casps7*, and observed reduced activation of autophagy upon silencing. Our results provide evidence that apoptosis-related genes are also involved in regulating autophagy, and that *Aedronc* may play an important role in DENV-2 infection success in *Ae. aegypti*, possibly through its regulation of autophagy.

Keywords: mosquito, *Aedes aegypti*, dengue virus, apoptosis, programmed cell death, autophagy, caspase, midgut, vector biology, host-pathogen interaction

Graphical abstract
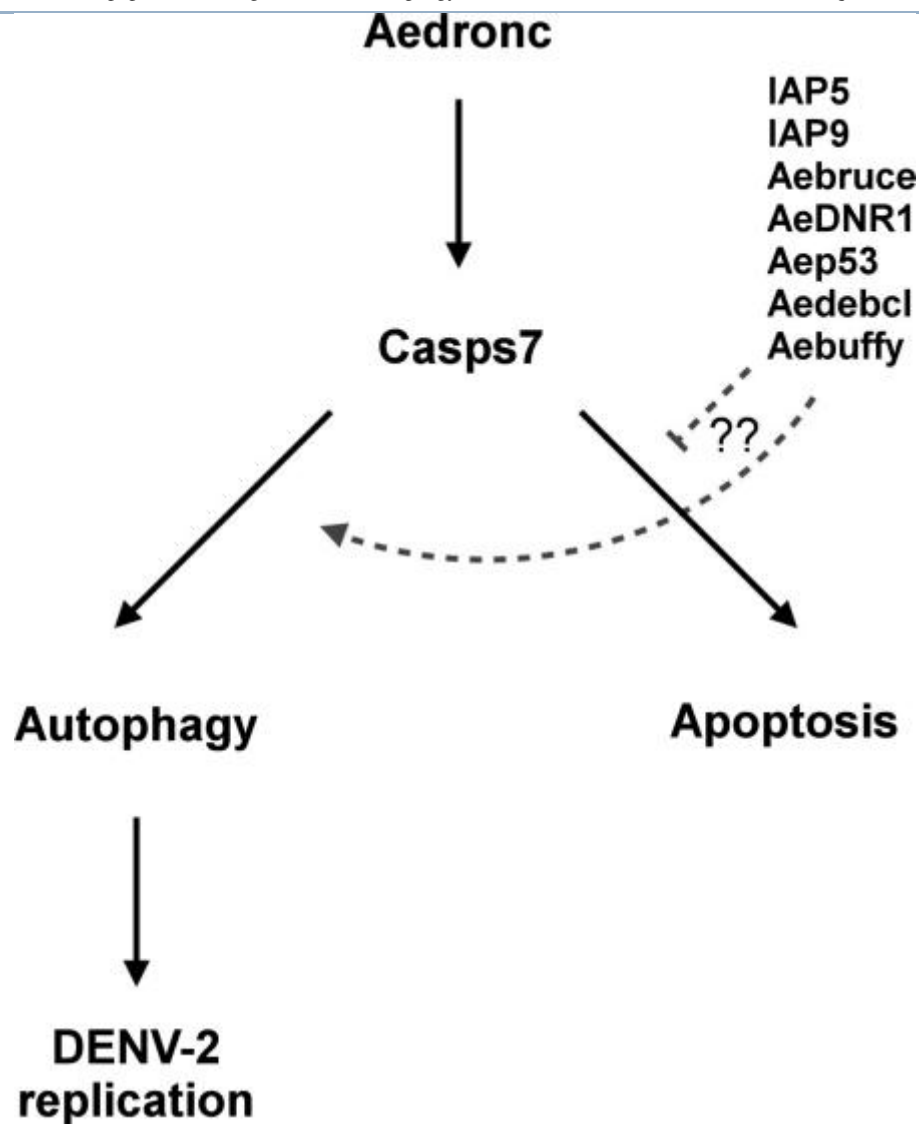
1. Introduction

Dengue fever, typically caused by one of four common serotypes of dengue virus (DENV 1-4), is one of the most medically important arthropod-borne diseases in the world. Much of the persistence and spread of DENV can be attributed to the expansion of its mosquito vectors, *Aedes aegypti* and *Aedes albopictus*. Although some recent advances have been made in the development of a vaccine (Thomas & Rothman, 2015), all current candidates are only partially effective (Guzman & Harris, 2015; Sabchareon *et al*, 2012). Mosquito control, therefore, remains the only viable option for preventing and containing transmission of DENV.

To become transmissible by a mosquito vector, DENV must successfully infect and replicate in the mosquito midgut epithelium, escape into the hemocoel and infect salivary gland tissues (reviewed in Black *et al*, 2002). Transcriptional analyses of female *Ae. aegypti* response following DENV-infected blood meals have identified multiple gene networks and pathways that represent potential molecular targets for blocking DENV transmission (Behura *et al*, 2011, 2014; Bonizzoni *et al*, 2012a; Chauhan *et al*, 2012; Colpitts *et al*, 2011; Sim *et al*, 2013). Our previous whole transcriptome study indicated that a group of genes associated with apoptosis showed significant coordinated upregulation in a DENV refractory strain in response to

DENV-2 infection (Behura *et al,* 2011). Apoptosis of mosquito salivary gland and midgut tissue has been associated with infection by multiple viruses including, West Nile virus, Sindbis virus (SINV), densovirus and nucleopolyhedrovirus (Kelly *et al,* 2012; Liu *et al,* 2011; Roekring & Smith, 2010; Vaidyanathan & Scott, 2006). Progressive salivary gland apoptosis has been correlated with decreased long-term West Nile virus titers in *Culex quinquefasciatus* (Girard *et al,* 2007).

Apoptosis is typically activated when stimuli, such as UV light, irradiation or developmental changes, induce activation of cysteine proteases known as caspases, which then cleave downstream proteins leading to cell death. In *Ae. aegypti,* the initiator caspase, *Ae*dronc (also known as CaspL2), auto-activates upon association to the adaptor protein Ark to form a complex known as the apoptosome. Activated *Ae*dronc then cleaves and activates the effector caspases, Casps7 and Casps8. The *Ae. aegypti* inhibitor of apoptosis 1 (*Ae*IAP1) binds and inhibits all three of these caspases, preventing spontaneous apoptosis. In addition, the IAP-antagonist protein Michelob_x (Mx) competitively binds to caspases and prevents their inhibition by *Ae*IAP1 (Liu & Clem, 2011; Wang & Clem, 2011).

In other arthropods, some proteins traditionally associated with apoptosis, also regulate the cell recycling mechanism known as autophagy (Baehrecke, 2005; Daish *et al,* 2004; Hou *et al,* 2008; Kim *et al,* 2010; Lee *et al,* 2003; Maiuri *et al,* 2007; Martin & Baehrecke, 2004). Autophagy is a highly conserved catabolic process activated in all eukaryotic cells to degrade unused or old organelles and cellular cargo, and is driven by a suite of core autophagy-related (ATG) proteins (Feng *et al,* 2014; McPhee & Baehrecke, 2009; Yorimitsu & Klionsky, 2005). During macroautophagy, a double membrane vesicle called an autophagosome forms around cytosolic components, which subsequently fuse with lysosomes and degrade its contents (Feng *et al,* 2014). Under certain conditions in *Drosophila*, midgut and salivary gland tissues show both high caspase activity and the formation of autophagosomes (Lee & Baehrecke, 2001; Lee *et al,* 2002; Martin & Baehrecke, 2004; Nezis *et al,* 2009; Velentzas *et al,* 2007), suggesting that apoptosis and autophagy may be highly integrated in arthropods. Mosquitoes are known to activate autophagy following vitellogenesis to recover excess yolk protein (Bryant & Raikhel, 2011; Mane-Padros *et al,* 2012).

Autophagy has been shown to be necessary for dengue infection in mammalian cells. DENV-infected murine cells show increased autophagosome formation, expression of LC3/ATG8 on autophagosomal membranes, and maturation of autophagosomes (Lee *et al,* 2008; Lee *et al,* 2013; McLean *et al,* 2011). Depletion of autophagy can decrease DENV titers over 1000-fold (Lee *et al,* 2008; Heaton & Randall, 2010; McLean *et al,* 2011; Mateo *et al,* 2013). However, whether autophagy functions similarly in invertebrate hosts has not yet been determined.

Here we investigated the role of cell death mechanisms in DENV infection of Ae. aegypti strains known to be refractory and susceptible to DENV infection. We observed increased apoptotic activity only in midguts of refractory strain females upon DENV-2 infection. However, RNAi-induced reduction of *Aedronc,* that should limit activation of apoptosis, unexpectedly resulted in decreased levels of DENV-2 infection. We also observed that following a naïve blood meal, females showed activated autophagy, as well as parallel upregulation of autophagic and apoptotic genes, including *Casps7*. RNAi-induced *Aedronc* reduction attenuated Casps7 upregulation as well as activation of autophagy. Further analysis revealed that Casps7 may also regulate autophagy, suggesting autophagy could play a similar role in DENV-2 infection in mosquitoes as has been demonstrated for humans.

2. Materials and Methods

2.1 Mosquito Rearing and Maintenance

Mosquitoes were reared and maintained according to standard laboratory protocol (Clemons et al, 2010). Similar numbers of larvae (~125) were grown in pans of distilled water and fed a slurry of bovine liver powder *ad libitum*. Pupae were collected and placed in 250 mL of distilled H_2O in 475 mL cups and allowed to emerge as adults in a 19 L plastic container with mesh top. Moyo-R (MR) and Moyo-S (MS) strains used in this study were previously derived from the Moyo-In-Dry strain, collected from Mombasa, Kenya in 1974. These strains were selected for refractoriness (MR) or susceptibility (MS) to the malaria parasite *Plasmodium gallinaceum* (Thathy et al, 1994), but were subsequently found to also show low frequency (~0.2) and high frequency (~0.54) of DENV-2 dissemination, respectively, when provided an infected blood meal (Schneider et al, 2007).

2.2 Virus Propagation and Blood feeding

Ae. albopictus C6/36 cells were cultured according to protocol in Schneider et al. (2007), with minor modifications. Cells were maintained in 75-cm^3 flasks with L-15 media (Lonza, Allendale, NJ) supplemented with 10% fetal bovine serum (FBS), at 28°C without CO_2. At ~85% confluency, cells were inoculated with DENV-2 (strain JAM1409, a high passage virus; Deubel et al, 1986) at a multiplicity of infection (MOI) of 0.1 and incubated with L-15 media with 2% FBS at 28°C witho ut CO_2. After 7 days, cells were centrifuged at 2500 RPM for 10 min and supernatant was collected for mosquito infections.

Mosquitoes were fed defibrinated sheep blood (Colorado Serum Company) mixed in a 1:1 ratio with either control C6/36 cell supernatant, or DENV-2 JAM1409 supernatant as previously described (Schneider et al, 2007). Briefly, blood feeding was performed using a glass membrane feeder attached to a circulating water bath that warmed blood to 37°C. Sausage casing was used as an artificial memb rane to cover the bottom of the feeder. Mosquitoes were allowed to feed for ~10 minutes before fully engorged females were CO_2-anesthetized and separated into 475 mL cups with 12-15 individuals each. Infective blood feeding for MS and MR strain females were always performed concurrently. Mosquitoes were maintained in an environmental chamber according to our standard laboratory protocol (Clemons et al, 2010) and fed a 5% sucrose solution *ad libitum*.

2.3 Gene knockdown by RNAi

To create dsRNA, total genomic DNA was extracted from two adult MR females using a simple, rapid alkaline method (Rubeck & Dissing, 1998). Primers with the T7 promoter sequence (5'-TAATACGACTCACTATAGGG-3') added to their 5' ends were used to amplify regions of *Aedronc, Casps7, ATG1, ATG5* and *ATG7* genes that showed little or no homology with other genes according to VectorBase (Supp. Table S1). *Aedronc* is necessary for apoptosis in *Ae. aegypti* (Liu & Clem, 2011; Wang & Clem, 2011), and a *Casps7* homolog was found to regulate autophagy is *Drosophila* (Hou et al, 2008; Kim et al, 2010). *ATG1*, *ATG5* and *ATG7* were chosen based on their known core roles in autophagy in

Drosophila (Chang *et al,* 2010). As a non-targeting control, *B-galactosidase* gene (*B-gal*) was amplified from pBluescriptIIKS plasmid (Berois *et al,* 2012). dsRNA was synthesized and purified from PCR product using the MEGAscript Kit (Ambion) according to manufacturer's protocol.

To deliver dsRNA, 2-3 day old cold-anesthetized adult female mosquitoes were injected in the thorax with ~69 nL of 2.3 μg/μL dsRNA using a micro-injector as previously described (Berois *et al,* 2012; Sim & Dimopoulos, 2010). Mosquitoes were then maintained in an environmental chamber and fed a 5% sucrose solution *ad libitum*. Gene knockdown was tested 48 h post injection (HPI) via quantitative PCR (qPCR) on pools of five mosquitoes, as described below. Mosquitoes used for DENV-2 infection studies were blood fed 48 HPI, as described above. Statistical analysis of *Aedronc* or *Casps7* knockdown was performed by Student's *t*-test to compare ds*Aedronc*- or ds*Casps7*-treated females, respectively, to ds*B-gal* treated females.

2.4 Quantitative PCR

To compare expression of apoptotic genes across time and between MR and MS strains, females were collected before feeding and every 24 h until 72 h PBM. To determine whether strains showed differential activation of apoptotic genes upon DENV-2 infection, we tested 5 pooled whole bodies at 24 and 48 h PBM and 10 pooled midguts at 48 h PBM from DENV-2 and naïve-fed MR and MS. To look at expression changes of autophagy and apoptosis-related genes after a blood meal, adult MR females were fed a naïve blood meal, and collected before feeding and every 12 h until 72 h PBM.

RNA was isolated using TRIzol reagent according to manufacturer's protocol (Invitrogen). RNA samples were DNA-digested using RQ1 DNAse I (Promega) and then cDNA was created using Superscript II Reverse Transcriptase (Invitrogen). Primers were designed for the amplification of apoptosis genes (*Aedronc*, *AeIAP1*, *Mx*), apoptosis-related genes (*Aep53*, *IAP5*, *IAP9*, *Aebruce*, *AeDNR1*, *Aedebcl*, *Aebuffy*, *Casps8*, *Imp*) and autophagy genes (*ATG1*, *ATG4A*, *ATG5*, *ATG8*, *ATG12*) (Supp. Table S2). Apoptosis-related genes were chosen based on their potential relationship to autophagy (Hou *et al,* 2008; Kim *et al,* 2010; Lee *et al,* 2003). This cDNA was used as template in real time quantitative PCR (qPCR), using Power SYBR Green One-Step (Applied Biosystems) for quantification. RNA expression was obtained using the ABI 7500 Fast System Sequence Detector System (Applied Biosystems). Delta Ct values (Schmittgen & Livak, 2008) for genes of interest were normalized against the housekeeping gene *ribosomal protein S17* (*RPS17*) (Morlais *et al,* 2003) or *ribosomal protein L8* (*RPL8*) (Lan & Fallon, 1992). Relative quantification (RQ) values were obtained using the delta-delta C_T ($\Delta\Delta C_T$) method.

Statistical analysis for RQ data was performed by either (A) two-way ANOVA to investigate effects of time PBM and strain on RQ of genes, followed by the Tukey honestly significant difference (HSD) test to identify significant pair-wise differences among means, (B) Student's t-test to compare ratios of *Aedronc*, *AeIAP1* and *Mx* transcript from DENV-fed to control-fed females between strains, or (C) one-way ANOVA to investigate effects of time PBM on expression of autophagy and apoptosis-related genes, followed by the Tukey HSD test to identify significant differences among means in expression relative to 0 h PBM.

2.5 TUNEL Staining

To test for apoptotic activity in response to DENV-2 infection, midguts from mosquitoes fed on control or DENV-2 infected artificial blood meals were dissected out in PBS at 48 h PBM and frozen in PBS at −80°C until staining. Mi dguts were whole mounted onto glass slides and then stained for fragmented DNA using the ApopTag Detection kit (Chemicon, Temecula, CA). Nuclei were stained with DAPI. Slides were immediately viewed using a Leica DM550 B Widefield Microscope and images were captured with a Hamamatsu ORCA-R2 C10600 Digital Camera. Slides were visualized at 40X and 2-3 images were taken from different sections of each midgut. TUNEL-positive cells were counted from each section and calculated as a proportion of the total number of cells visible within the midgut. Statistical analysis comparing midgut proportion of TUNEL-positive cells between groups was performed using a Mann-Whitney U Test.

2.6 RT-PCR

To determine whether DENV-2 blood fed females showed disseminated infection from the midgut, total RNA was isolated from homogenized individual leg tissue of females 14 d PBM using TRIzol reagent. Virus was detected using DENV-specific primers that amplified a 511 bp region of the genome (Lanciotti et al, 1992), and multiplexed with primers for *ribosomal protein L8* (*RPL8*) as a control (Lan & Fallon, 1992). cDNA was amplified using the Superscript III One-Step RT-PCR kit (Invitrogen) according to manufacturer's protocol. The proportion of positive leg samples was obtained for each of three biological replicates. Proportion data was arcsin transformed and then analyzed for significance using Student's *t*-test.

2.7 Virus Titration

Virus titer was quantified from individual females fed a DENV-2 infected blood meal 14 d PBM, as well as from the artificial blood meals used for feeding, using the $TCID_{50}$ method (Nawtaisong et al, 2009). To quantify virus in infected mosquitoes, single mosquitoes were ground in 1 mL of Schneider's media (Lonza) on ice and the triturate was then filtered through 0.45 μm filters. 100 μL of filtered triturate was then 10-fold serially diluted in 2% FBS L-15 media (Lonza) in 96-well plates, and then an equal volume of C6/36 cells was added to each well. For quantification of DENV-2 from artificial blood meals used for infection, 100 μL of the blood meal was serially diluted as described above. Cells were incubated for 4-5 days before fixation with 3:1 acetone:PBS solution. DENV was detected using the DENV-2 antibody 2B29A (Santa Cruz Biotechnology) in a 1:100 dilution, followed by 1:100 sheep anti-mouse biotinylated IgG (GE Healthcare) and addition of 1:100 Strepavidin Alexafluor-680 (Invitrogen). Plates were imaged using a LI-COR scanner. Cells showing cytoplasmic fluorescence were scored as positive for infection. The $TCID_{50}$ was calculated using the Reed-Muench method (Reed & Muench, 1938). The titers of DENV-2 within blood meal ranged from $10^{6.3}$-$10^{7.7}$ TCID50/mL. Statistical analysis comparing virus titers was performed using a Mann-Whitney U test.

2.8 Western Blots

Adult MR females were injected with dsRNA as above. Due to the expected constant flux of autophagy, females were also co-injected with 10 mM hydroxychloroquine (HCQ) to block degradation of autolysosomes (Barth et al, 2010; Kroemer & Jäättelä, 2005; Rubinsztein et al, 2009). To determine whether programmed autophagy was caspase-dependent in fat body, females were fed a control artificial blood meal as above, and at 36 h PBM five midgut-free carcasses were collected for protein extraction, as most autophagy

genes were previously shown to have maximum transcription at this time point (Bryant & Raikhel, 2011). To determine whether DENV affected autophagy, and whether autophagy was caspase-dependent in mosquito midgut, females were fed either a control or DENV-2 infected artificial blood meal as above. To allow sufficient time for virus to replicate (Salazar *et al*, 2007), 10 midguts were dissected out in 1X PBS at 3 d PBM, and stored in PBS at $-80°C$ until protein extraction. Approximately 14 ng of total protein was size-fractionated on Tris-Glycine gels (Pierce) and then transferred to PDVF membrane. ATG8 was detected using polyclonal rabbit anti-mosquito ATG8 at a 1:1500 dilution (Bryant & Raikhel, 2011), followed by secondary anti-rabbit-HRP antibody (Life Technologies) at 1:2500 dilution. α-tubulin was used as a loading control at a 1:10,000 dilution followed by secondary anti-mouse antibody at a 1:10,000 dilution (Santa Cruz Biotechnology). ATG8 is normally cytoplasmic, but upon activation of autophagy, is conjugated to phosphatidyl ethanolamine (PE) on the autophagosomal membrane through a series of ubiquitination cascades (Klionsky *et al*, 2012). ImageJ was used for densitometry analysis of a representative western blot comparing the ratio of total ATG8 protein to α-tubulin or ratio of ATG-PE to ATG8. Statistical analysis was performed using either: (A) one-way ANOVA to investigate effects of treatment on ratio of total ATG8 protein to α-tubulin or ratio of ATG-PE to ATG8, followed by the Tukey HSD test to identify significant pair-wise differences relative to ds*B-gal*-treated samples at 36 h PBM, or (B) Student's *t*-test.

2.9 Lysotracker Staining

Lysotracker is an acidotropic dye used as a marker for autolysosome formation (Bryant & Raikhel, 2011; Klionsky *et al*, 2012; Mauvezin *et al*, 2014). To determine whether autophagosomes were present in individual tissues, midguts and fat body were dissected out individually before blood feeding and 36 h PBM in 1X PBS and then stained with 0.5 µM LysoTracker Red (Invitrogen) for 5 min. DAPI was used as a DNA counterstain. Slides were immediately viewed using a Leica DM550 B Widefield Microscope and images were captured with a Hamamatsu ORCA-R2 C10600 Digital Camera. Slides were visualized at 100-200X.

3. Results

3.1. Refractory mosquitoes show increased midgut epithelial cell death following DENV infection

In order to determine transcriptional activity of apoptotic genes following a blood meal, we measured whole body transcriptional activity of *Aedronc*, *AeIAP1* and *Mx* in MR and MS females 0-72 h following a naïve blood meal. MR females showed a 2.5-fold increase in *Aedronc* expression at 48 h PBM (p=0.005), which then declined by 72 h (p=0.015) (Fig 1A). In contrast, MS females showed a 2.1-fold, albeit insignificant (p=0.050), increase at this time. MR also showed a significant 1.8-fold increase (p=0.026) of *AeIAP1* expression (Fig 1B) by 48 h, while MS did not show a significant (p=0.763) increase. *Mx* expression was elevated 5.6-fold in the MR strain by 48 h (p=0.002), but only 2.0-fold in the MS strain (p=1.000), compared to 0 h PBM (Fig 1C). At 48 h, MR females had significantly increased *Mx* compared to MS females (p=0.003). Thus, MR showed upregulation of *Aedronc*, *AeIAP1* and *Mx* following a blood meal. In addition, at 48 h PBM, *Mx* was significantly upregulated in MR compared to the MS strain.
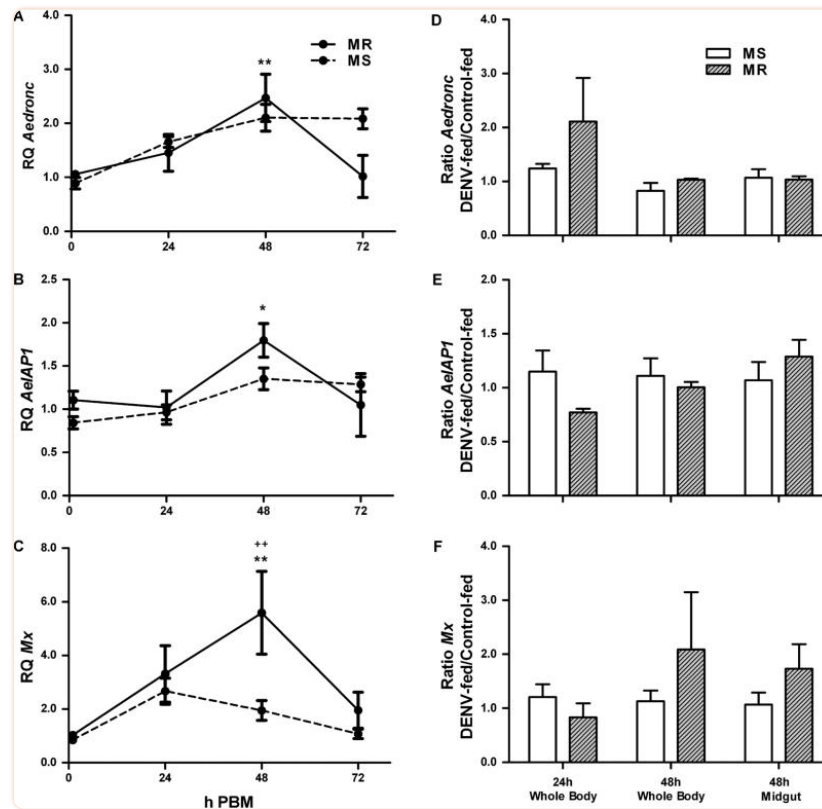
[Fig 1](#)

Apoptotic gene expression following a DENV-infected blood meal

(A-C) MS (susceptible strain) and MR (refractory strain) females 4-5 days post eclosion were fed a naïve artificial blood meal and qPCR was performed on RNA extracted from whole mosquitoes collected at 0, 24, 48, and 72 h post blood meal (PBM). Expression of (A) *Aedronc*, (B) *AeIAP1*, and (C) *Mx* was analyzed. Expression is represented as Relative Quantitation (RQ) compared to samples collected before a blood meal. Apoptosis genes showed significant transcriptional upregulation following blood meal compared to females 0 h PBM. Each data point represents mean expression (n=3 replicates; pools of 5 whole mosquitoes per replicate). (D-F) 2-3 day old adult MS and MR females were fed either control or DENV-2 JAM1409-supplemented artificial blood meals. At 24 and 48 h PBM, either whole mosquitoes or midguts were collected and tested for expression of (D) *Aedronc*, (E) *AeIAP1*, or (F) *Mx* transcript. Data represent ratio of transcript of DENV-2-fed to control-fed females (n=3-5 replicates; individual replicates represent the mean of 5 pooled whole females or 10 pooled midguts). Error bars represent standard errors of means. Statistical comparisons were performed using (A-C) two-way ANOVA followed by the Tukey HSD test to identify significant PBM differences relative to 0 h PBM or (D-F) Student's *t*-test to compare ratios of transcript of DENV-2-fed to control-fed females between MS and MR. *$p<0.05$; **$p<0.01$ within strain comparison between 0 h PBM and time point. ++$p<0.01$ between strain comparison.

To determine whether apoptotic gene expression was changed upon DENV-2 infection, we measured whole body transcription levels of *Aedronc*, *AeIAP1* and *Mx* in MS and MR females following a naïve or DENV-2-infected blood meal. We did not find any statistically significant differences in the expression of *Aedronc*, *AeIAP1* or *Mx* due to infection ([Fig 1E-F](#); [Supp. Table S3](#)). However, at 24 h PBM *Aedronc* was elevated in DENV-fed MR compared to DENV-fed MS ([Fig 1D](#)), and at 48 h, *Mx* was ~2.0-fold greater in

DENV-fed MR compared to DENV-fed MS, but highly variable between replicates (Fig 1F). We also looked at transcripts of *Aedronc*, *AeIAP1* and *Mx* in the midguts of MS and MR mosquitoes at 48 h PBM (Fig 1D-F). Again, there were no statistically significant differences for *Aedronc*, *AeIAP1* or *Mx* upon infection or between strains. However, *Mx* again showed slightly higher expression in infected MR midguts (Fig 1F), as observed previously in whole bodies, which may suggest elevated apoptotic activation in refractory mosquitoes in response to DENV.

We then assessed apoptotic activity in midgut cells from MS and MR 48 h PBM by TUNEL staining. We observed a basal level of apoptosis occurring in the midguts of naïve-fed females in both strains, with no significant differences between strains (p=0.167). Upon challenging MS and MR with DENV-2, MS midguts did not show differences in TUNEL-positive cells compared to control midguts. However, DENV-2 infected MR midguts showed a significant increase from 0.26% to 0.45% (p=0.0388) of cells being TUNEL-positive compared to control-fed MR midguts (Fig 2A-C). In addition, DENV-2 infected MR midguts also reflected a significantly higher percentage of TUNEL-positive cells than those from control or DENV-2 infected MS midguts (p=0.0055; p=0.0074, respectively) (Fig 2D). We did not observe any obvious bias for anterior or posterior localization of apoptotic foci under any of the conditions tested. Thus, refractory mosquitoes showed significantly more apoptotic activity in midgut tissue upon DENV-2 exposure across all comparisons.
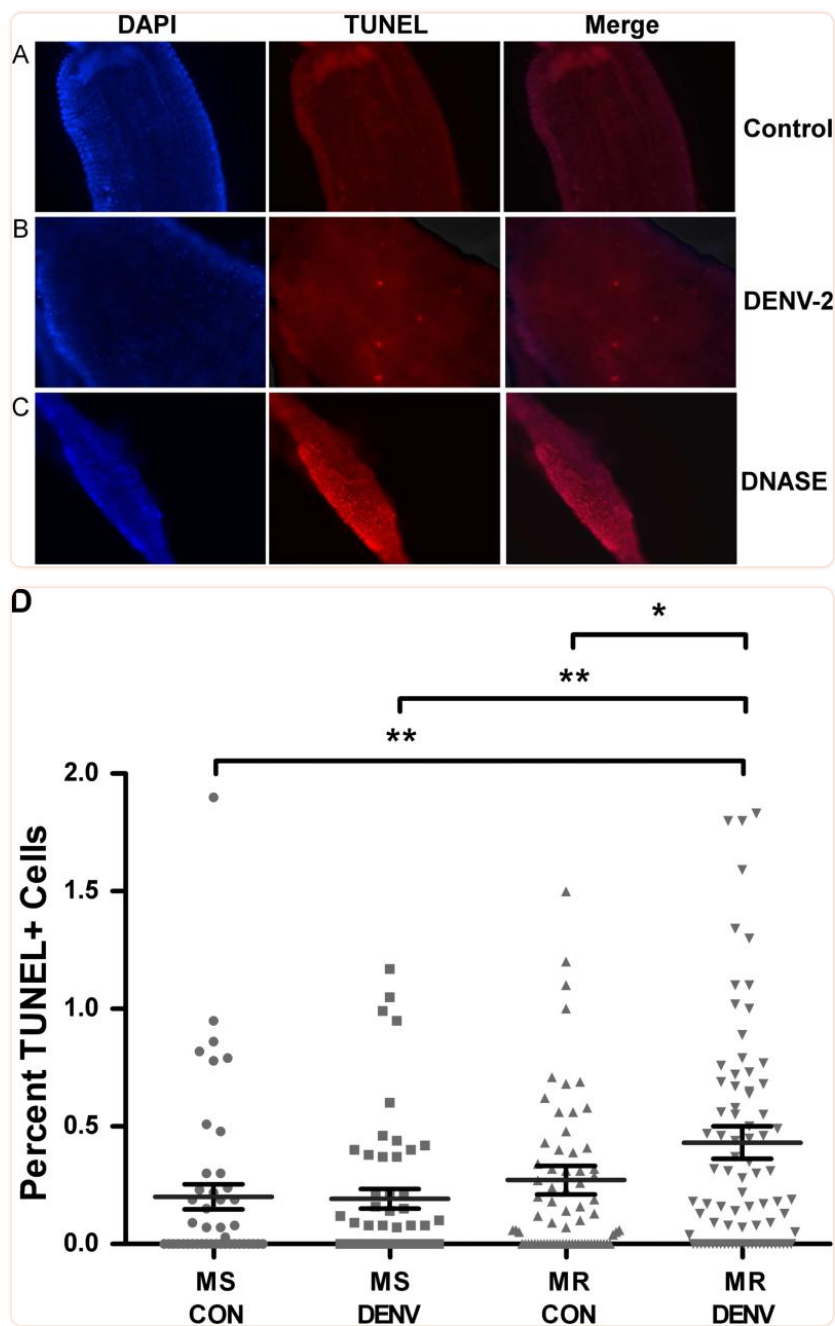
[Fig 2](#)

DENV-2-infected midguts from refractory strain (MR) of *Ae. aegypti* show increased apoptotic cells

MR and MS females 4-5 days post eclosion were fed either (A) control or (B) DENV-2 JAM1409-supplemented artificial blood meals. At 48 h PBM midguts were dissected out and nuclei were stained with DAPI (blue) and apoptotic cells were stained by TUNEL (red). (C) As a positive control, midguts were incubated with DNAse for 30 min and then stained for TUNEL. Depicted images are of MR mosquitoes. Magnification at 40X. (D) Apoptotic nuclei were quantified as the proportion of the total number of cells in each midgut section (n=3 replicates; 16-28 sections per independent replicate). Statistical analysis of percent midgut cells staining TUNEL+ between groups was performed using Mann-Whitney U test. *p<0.05; **p<0.01.

3.2. Knockdown of Aedronc decreases DENV infection

To determine whether apoptosis contributed to DENV susceptibility, we knocked down *Aedronc* in MR females. Females injected with ds*Aedronc* showed ~40% reduction in *Aedronc* transcript by 48 HPI (Fig 3A). Knockdown of *Aedronc* significantly decreased the percentage of mosquitoes showing disseminated infection from 22.1% to 7.3% (p=0.0236) (Fig 3B). Mosquitoes showing disseminated infection and reduced for *Aedronc* also showed a half-log reduction in DENV titer from $TCID_{50}$ $10^{2.4}$ to $10^{1.6}$ (p=0.0204) (Fig 3C). Thus, *Aedronc* knockdowns resulted in both decreased dissemination frequencies and virus titer in those individuals that were infected. In combination, these experiments show that *Aedronc* is important for DENV-2 infection and may play a positive role in the ability of the virus to replicate in or infect cells.
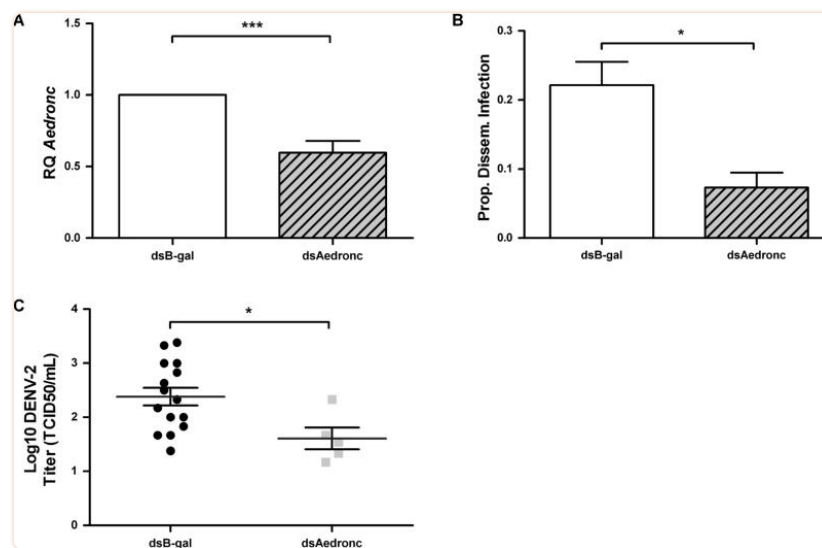

Fig 3

***Aedronc* knockdown decreases the proportion of mosquitoes infected and DENV-2 titer among infected individuals**

Refractory (MR) females 2-3 days post eclosion were injected with either ds*Aedronc* or ds*B-gal* and: (A) tested for knockdown of *Aedronc* by qRT-PCR 48 h post injection (HPI). Expression is represented as Relative Quantitation (RQ) compared to ds*B-gal*-treated females (n=5 replicates; 5 pooled females per replicate). (B) 48 HPI, females were provided DENV-2 JAM1409-supplemented artificial blood meals. Mosquitoes were collected at 14 d PBM and tested for disseminated infection by RT-PCR of DENV RNA from leg tissue (n=3 replicates; no. infected females/total tested per replicate: B-gal: 7/30; 12/44; 9/57; *Ae*dronc: 4/38; 3/36; 1/32). (C) Mosquitoes were homogenized in cell media, and infectious titer was measured by $TCID_{50}$ assay (n=2 replicates). Error bars represent standard errors of means from biological replicates. Statistical analyses were performed using (A-B) Student's t-test or (C) Mann-Whitney U test. *p<0.05; ***p<0.001.

3.3. Autophagy is upregulated following blood feeding

To analyze the possibility that apoptosis genes are involved with regulation of autophagy in *Ae. aegypti*, we assessed whether programmed autophagy occurs post blood meal. Transcriptional activity of the ATG genes, *ATG1*, *ATG4A*, *ATG5*, *ATG8* and *ATG12* was elevated in whole MR females following a naïve blood meal compared to sugar fed mosquitoes ([Fig 4](#)). Each gene showed statistically significant increases in expression following a blood meal, and we observed significant differences in expression between time points as well ([Supp. Table S4](#)). Expression of *ATG1*, *ATG4A*, *ATG5* and *ATG12* showed the greatest increases in expression at 36 h, with relative expression 2.9-3.6-fold higher (p=0.0011; p=0.0002; p=0.0038; p=0.0023) than that of sugar-fed mosquitoes. *ATG8* peaked at 48 h with 2.3-fold greater expression (p=0.0314) than sugar-fed mosquitoes. To confirm autophagy is activated 36 h PBM, we performed western blotting of ATG8. We observed that females showed non-lipidated ATG8 prior to blood feeding. However at 36 h PBM, we observed an increase in ATG8-PE and concordant reduction in non-lipidated ATG8 ([Fig 5A](#)), thus indicating activation of autophagy. To visualize where autophagic vesicles occurred, we stained fat body and midgut tissues with the acidotropic dye, Lysotracker Red. Here, we found that both fat body and midgut tissue were devoid of Lysotracker staining before feeding, but showed bright puncta at 36 h PBM, further indicating activation of autophagy at that time. ([Fig 5B](#)). Taken together, these data confirm that autophagy is active at 36 h PBM in naïve-fed MR females.
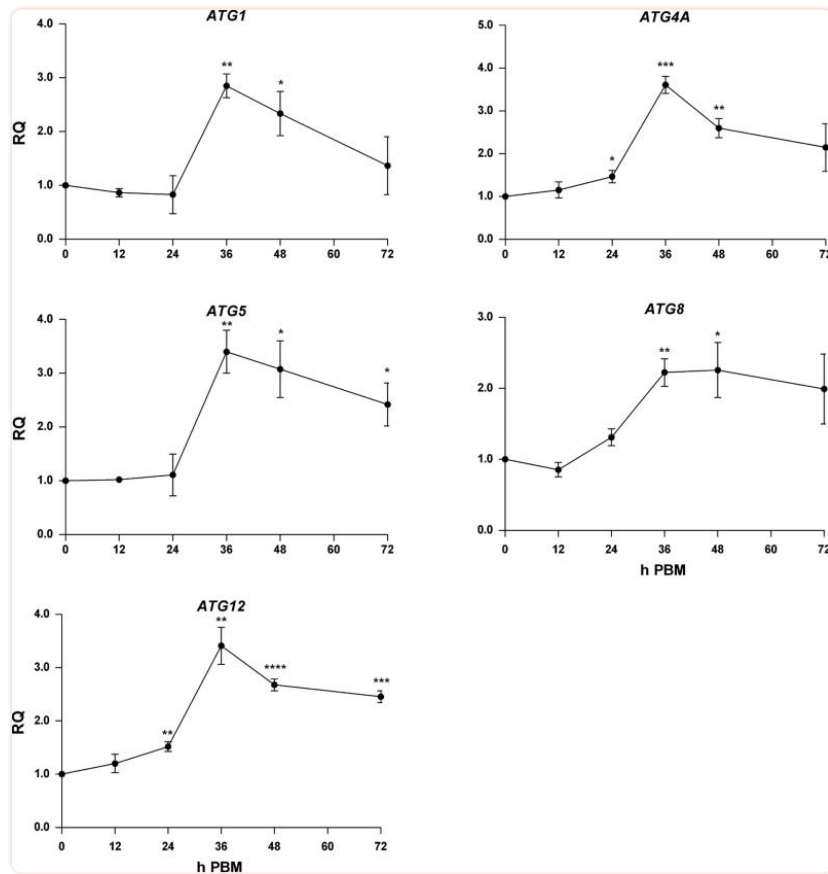
[Fig 4](#)

Autophagy genes are upregulated following a blood meal

MR females 4-5 days post eclosion were fed a naïve artificial blood meal and qPCR was performed on RNA extracted from whole mosquitoes collected at 0, 12, 24, 36, 48, and 72 h PBM. Expression of the autophagy genes *ATG1*, *ATG4A*, *ATG5*, *ATG8* and *ATG12* was analyzed. Expression is represented as Relative Quantitation (RQ) compared to samples collected before a blood meal. Autophagy genes showed significant upregulation following blood meal compared to females fed 0 h PBM. Each data point represents mean expression (n=3 replicates; pools of 5 whole mosquitoes per replicate). Error bars represent standard error of means. Statistical comparisons were performed using one-way ANOVA followed by the Tukey HSD test to identify significant PBM differences relative to 0 h PBM. *$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$
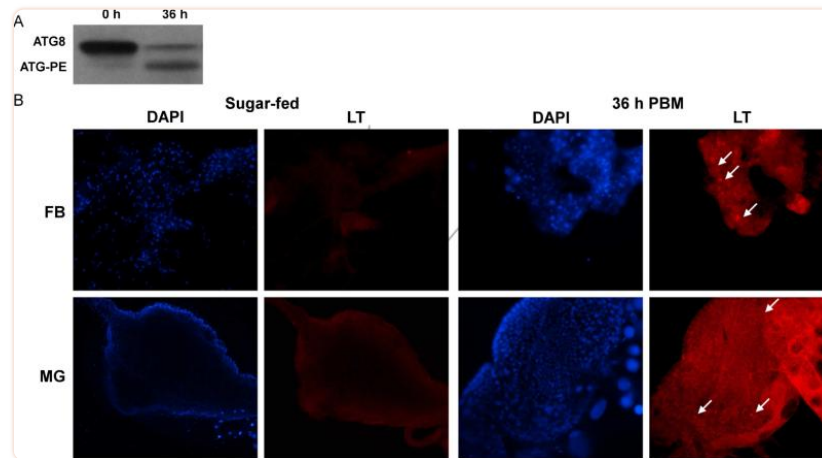
[Fig 5](#)

ATG8-PE conjugation and Lysotraker Red staining indicate autophagosome biogenesis following a blood meal

(A) MR females 4-5 days post eclosion were fed a naïve artificial blood meal. At 0 and 36 h PBM, pools of 5 whole females were tested for presence of the autophagy marker ATG8. ATG8 is cytoplasmic, but becomes conjugated to phosphatidyl-ethanolamine (PE) on the autophagosomal membrane during autophagy. (B) Female fat body and midgut tissues from mosquitoes fed only on sugar water and from mosquitoes 36 h PBM were stained with DAPI and Lysotracker Red. White arrows point to puncta representative of autophagosomes. Images are at 100X magnification.

3.4. Apoptosis-related genes are activated during autophagy

To determine whether Casps7 could be involved with autophagy, we assessed its expression following a naïve blood meal. Expression increased following a blood meal starting at 12 h PBM, and continuing to rise to 7.6-fold until 48 h PBM (p=0.0079). By 72 h PBM, transcript levels had begun to decline ([Fig 6](#)). We then monitored expression of other apoptosis-related genes post blood meal. *Aep53, IAP5, IAP9,* and *Aebruce* were significantly upregulated following a naïve blood meal, and had similar temporal profiles that coincided with the activation of autophagy ([Fig 6](#); [Supp. Table S5](#)). Expression was increased at least 3.4-fold following a blood meal, suggesting that apoptosis-related proteins could be associated with other physiological processes related to blood feeding. *AeDNR1, Aedebcl* and *Aebuffy,* did not show statistically significant differences in expression, but *AeDNR1* and *Aedbcl* showed patterns of upregulation by 36 h PBM, and *Aebuffy* showed a pattern of downregulation from 12-24 h PBM. Expression of *Casps8* and *Imp* was unchanged (data not shown). Thus several apoptosis-related genes show expression profiles paralleling programmed autophagy after blood feeding.
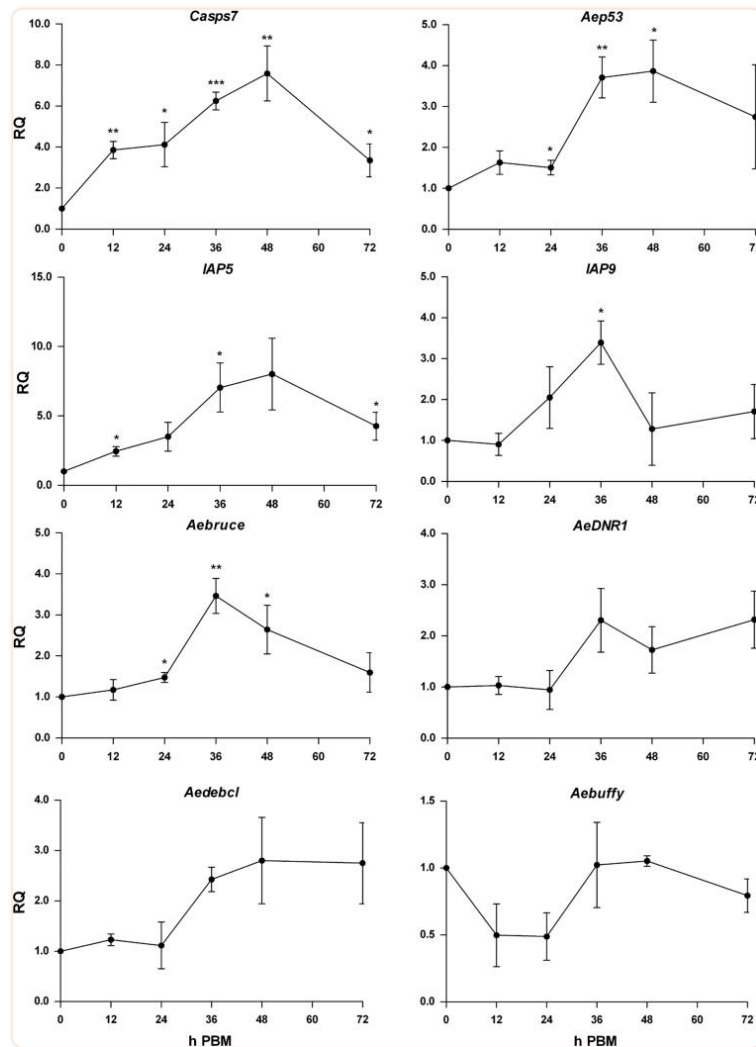
Fig 6

Upregulation of apoptosis-related genes post blood meal coincides with autophagy activation

MR females 4-5 days post eclosion were fed a naïve artificial blood meal and qPCR was performed on RNA extracted from whole mosquitoes collected at 0, 12, 24, 36, 48, and 72 h PBM. Expression of multiple apoptosis-related genes were analyzed, including *Casps7*, *Aep53*, *IAP5*, *IAP9*, *Aebruce*, *AeDNR1*, *Aedebcl*, and *Aebuffy*. Expression is represented as Relative Quantitation (RQ) compared to samples collected before a blood meal. Apoptosis-related genes showed significant transcriptional upregulation following blood meal compared to females 0 h PBM. Each data point represents mean expression (n=3 replicates; pools of 5 whole mosquitoes per replicate). Error bars represent standard error of means. Statistical comparisons were performed using oneway ANOVA followed by the Tukey HSD test to identify significant PBM differences relative to 0 h PBM. $*p<0.05$; $**p<0.01$; $***p<0.001$.

3.5. Aedronc and Casps7 genes regulate autophagy

To determine whether *Casps7* expression was affected by *Aedronc* knockdown in MR females, we analyzed *Casps7* transcription following ds*Aedronc* treatment ([Fig 7](#)). *Aedronc* knockdown did not affect transcript levels of *Casps7* before a blood meal. However, at 36 h PBM, we observed a significant reduction in

Casps7 expression following *Aedronc* reduction (p=0.0089) that represented ~60% of the transcription levels found in *dsB-gal* treated individuals. Thus, *Aedronc* may affect transcriptional activation of *Casps7* during blood feeding. We assessed expression of several ATG genes at 36 h PBM (*ATG1*, *ATG4A*, *ATG5*, *ATG8* and *ATG12*) post *Aedronc*-reduction, but did not find evidence that *Aedronc* knockdown attenuated transcriptional activation for any of these genes (data not shown).
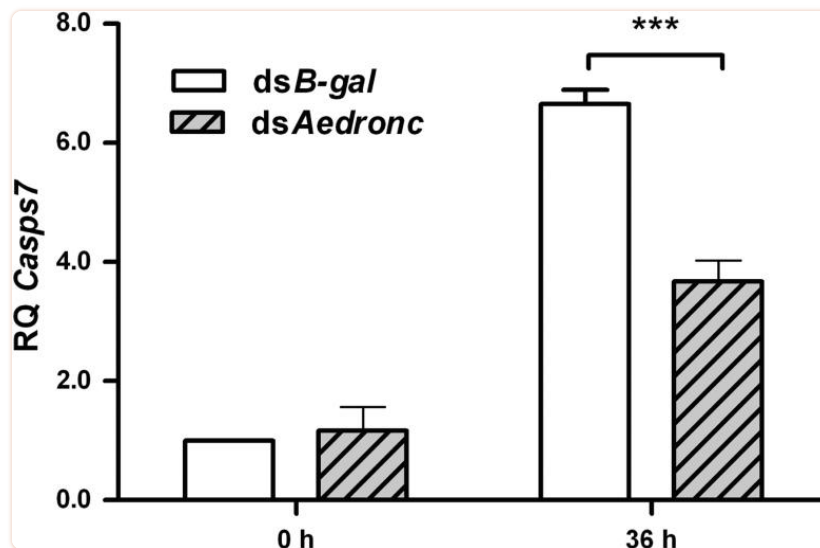

[Fig 7](#)

Aedronc regulates *Casps7* transcription following a blood meal

MR females 2-3 days post eclosion were injected with either ds*Aedronc* or ds*B-gal*, and then fed a naïve artificial blood meal 48 HPI. Females were collected at 0 and 36 h PBM. Total RNA was extracted and transcript from *Casps7* was quantified via qRTPCR. *Aedronc* knockdown decreased *Casps7* transcript following a blood meal, but not before. Expression is represented as Relative Quantitation (RQ) compared to ds*B-gal*-treated females. Each data point represents mean expression (n=3-5 replicates; pools of 5 whole mosquitoes per replicate). Error bars represent standard error of means. Statistical comparisons were performed using Student's *t*-test. *** p<0.001.

We then assessed the effect of *Aedronc* and *Casps7* reduction on autophagy in midgut-free carcasses of MR females. Naïve blood-fed *dsB-gal* females showed significantly higher levels of total ATG8 at 36 h PBM compared to *dsB-gal* females fed only on sucrose solution (p<0.0001) ([Fig 8A-B](#)), indicating that ATG8 is induced upon blood feeding. ds*Aedronc*-treated females showed a small but non-significant (p=0.077) reduction in total ATG8. However, midgut-free carcasses from ds*Casps7*-treated females showed a significant reduction of total ATG8 compared to ds*B-gal*-treated females (p=0.005). *ATG1*-silenced females showed near absence of total ATG8 (p=0.001), suggesting ATG1 is necessary for the production or stabilization of ATG8 in fat body. We did not observe differences in the ratios of ATG8-PE/ATG8 across treatments (not shown). We also assayed for differences in Lysotracker staining both in fat body and midgut tissue. Midguts and fat body from ds*B-gal*-treated females showed consistently bright

puncta. Upon dsRNA-mediated depletion of *ATG1*, *ATG5* and *ATG7*, in parallel with depletion of *Casps7,* both tissues showed absence of Lysotracker puncta ([Fig. 8C](#)). These experiments suggest that in midgut-free carcasses, Casps7 is important for autophagy, but *Ae*dronc may have a more limited role.
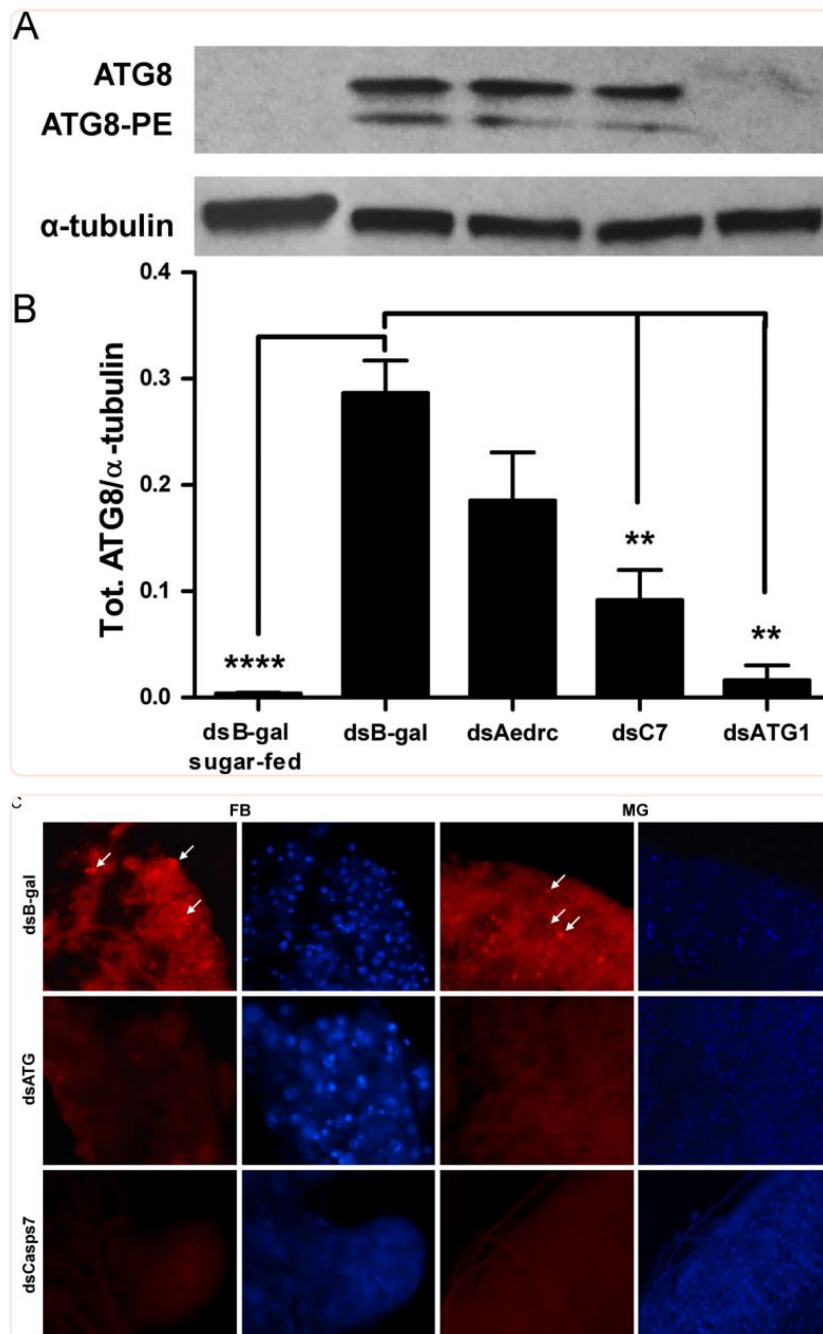
Fig 8

Casps7 regulates autophagy activation in fat body

(A) MR females 2-3 days post eclosion were injected with ds*Aedronc*, ds*Casps7*, ds*ATG1* or ds*B-gal,* and then fed a naïve artificial blood meal 48 HPI. 36 h PBM, midguts were dissected out and remaining bodies without midguts were tested for ATG8 by western blot. (B) Densitometry analysis was used to quantify the ratio of total ATG8 to α-tubulin (n=3-4 replicates; 5 pooled bodies without midguts per replicate). Error bars represent standard error of means. Statistical comparisons were performed using oneway ANOVA followed by the Tukey HSD test to identify significant differences compared to

ds*B-gal*-treated sample 36 h PBM. ** p<0.01; **** p<0.0001. (C) Females were treated with either ds*Casps7*, a cocktail of ds*ATG1*, ds*ATG5* and ds*ATG7* (*dsATG*), or ds*B-gal* and then fed a naïve blood meal as above. 36 h PBM, fat body and midguts were dissected out and stained with DAPI and Lysotracker Red. Images represent magnification at 200X.

To determine whether *Aedronc* or *Casps7* is involved in regulating autophagy in the midgut and whether autophagy is affected by DENV-2 infection, we assessed midgut tissue from MR females fed a DENV-2 infected blood meal. Females fed a control naïve blood meal showed a small, but non-significant (p=0.245), 1.9-fold increase in total ATG8 compared to females fed only on sucrose solution ([Fig 9A](#)). When compared to the increase of total ATG8 observed in midgut-free carcasses between sugar- and blood-fed females ([Fig 8B](#)), this suggests that there is a basal level of autophagy occurring in midgut tissue. Females challenged with DENV-2 did not show significant differences compared to those fed naïve blood. Total ATG8 was significantly reduced in DENV-2 fed females following knockdown of *Aedronc* (p=0.009), *Casps7* (p=0.0133) or triple depletion of *ATG1*, *ATG5*, and *ATG7* compared to DENV-2 fed females treated with ds*B-gal* (p=0.0003) ([Fig 9A](#)). Thus, in midgut tissue, both *Ae*dronc and Casps7 are necessary for autophagy. We did not observe differences in the ratios of ATG8-PE/ATG8 across treatments (not shown). Taken together, these experiments suggest that *Ae*dronc and Casps7 may act as positive regulators of autophagy, albeit with tissue-specific differences. While *Casps7* knockdown appears to be essential for autophagy activation in both fat body and midgut, *Aedronc* may primarily affect autophagy regulation in midgut tissue.
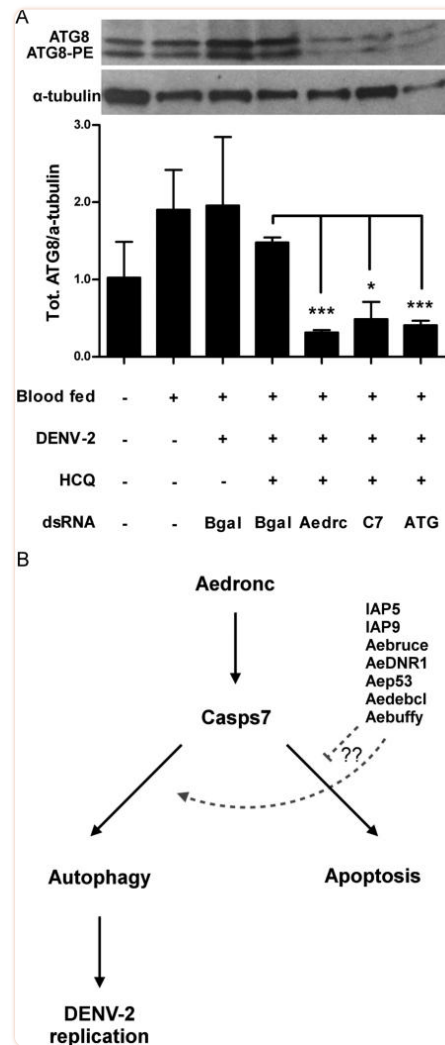
Fig 9

Aedronc and _Casps7_ knockdown reduces autophagy in midgut

(A) MR females 2-3 days post eclosion were co-injected with hydroxychloroquine (HCQ), and ds*B-gal*, ds*Aedronc*, ds*Casps7* or mixture of ds*ATG1*, ds*ATG5* and ds*ATG7 (ATG)*. 48 HPI, mosquitoes were fed either a control or DENV-2 infected artificial blood meal. At 3 d PBM, midguts were dissected out and analyzed for ATG8 by western blot. Densitometry analysis was used to quantify the ratio of total ATG8 to α-tubulin. (n=3-4 replicates; 10 pooled midguts per replicate). Total ATG8/α-tubulin was reduced in midguts of mosquitoes co-injected with HCQ and ds*Aedronc*, ds*Casps7* or ds*ATG* compared to midguts from those treated with HCQ and ds*B-gal*. Data represent means of replicates. Error bars represent standard error of means. Statistical comparisons were performed using Student's *t*-test of ratios of total ATG8/α-tubulin between groups * $p<0.05$; *** $p<0.001$. (B) Model for caspase control of autophagy. Following a blood meal, *Ae*dronc is needed for transcriptional activation of or stability of *Casps7* transcript. Casps7 can activate apoptosis or autophagy, but is guided to activate autophagy following a blood meal, possibly with the help of other apoptosis-related proteins. Autophagy may be associated with DENV-2 infection of midgut cells wherein it attenuates the apoptotic program and increases infection.

4. Discussion

4.1 Apoptosis in DENV infection

In this study we found that knockdown of *Aedronc* decreased DENV-2 infection in our refractory *Ae. aegypti* strain. In contrast, Ocampo *et al* (2013) previously reported that silencing *Aedronc* increased mosquito susceptibility to DENV. There are several possible explanations for this discrepancy. However, this simply may reflect that activation of apoptosis is complex and can be triggered through multiple pathways depending on experimental conditions such as MOI and tissue type (Klomporn *et al*, 2011; McLean *et al*, 2011; Pattanakitsukul *et al*, 2007; Theparrit, *et al*, 2013; Zargar *et al*, 2011). We note that in Ocampo *et al* both the range of titers of DENV used for infection and corresponding oral susceptibility seen with their refractory and susceptible strains were higher than those used in our study. Further, Ocampo *et al* primarily used immunofluorescence of head and midgut tissue to detect virus, while our study focused on detection of DENV RNA from leg tissue and virus titer. Therefore, it can be difficult to directly compare results across studies.

Another report showed that silencing *Aedronc* reduced the intensity of SINV infection in *Ae. aegypti* midgut and salivary glands, similar to our findings (Wang *et al*, 2012). SINV is well known to attenuate apoptosis in insects, and the finding that silencing this gene decreased infection suggests caspases may be multi-functional. However, a subsequent report by the same group (O'Neill *et al*, 2015) showed that although recombinant SINV-induced overexpression of Drosophila *Rpr* did initially induce strong apoptosis, they observed strong selection for null mutation viruses and an associated reduction in apoptosis. Results from these reports may be explained by difference in experimental approach and the genes targeted. O'Neill *et al* (2015) focuses on upstream activation of apoptosis by massive overexpression of *Rpr*, while the study by Wang *et al* (2012) utilized knockdown of *AeIAP1* and *Aedronc*. Downstream apoptotic effectors, such as *Ae*dronc and Casps7 could operate within both processes dependent on context, while upregulation of *Mx* may favor a pro-apoptotic outcome.

From our study it is unclear whether apoptosis or just caspase function is actually important for infection. Apoptosis is activated in response to infection with all four serotypes of DENV in human cells, through both intrinsic and extrinsic pathways (Klomporn *et al*, 2011; Liao *et al*, 2010; Nasirudeen *et al*, 2008; Umareddy *et al*, 2007). Furthermore, DENV has mechanisms to attenuate the apoptotic program (Lee *et al*, 2005; Li *et al*, 2012, McLean *et al*, 2011), suggesting apoptosis could also limit infection in the mosquito. Consistent with these studies, we observed increased apoptosis in MR midguts following a DENV-infected blood meal. However, we also observed programmed autophagy at this time. In addition, we and others (Bonizzoni *et al*, 2012a; Puglise *et al*, 2015) observed upregulation of several apoptosis-related genes, some of which control autophagy in insects (Accorsi *et al*, 2015; Denton *et al*, 2012b). While these genes may indeed be activated in response to pathogen infection, our finding that *Aedronc* knockdown reduces infection suggests that apoptosis-related genes may have additional roles. Furthermore, we found that both pro- (*Casps7, Aedronc*, *Mx, Aedbcl*) and anti-apoptotic (*AeIAP1, IAP5, IAP9, Aebruce*) genes were upregulated PBM, complicating the interpretation of how apoptosis, autophagy, or interaction between the two processes influences infection. In conjunction with other studies, it is clear that apoptosis is a complex process whose proteins may also function non-apoptotically depending on context.

4.2 Caspase control of autophagy

Autophagy is generally considered a pro-survival mechanism, while apoptosis is considered pro-death. However, in certain circumstances, cell death genes have been shown to have non-apoptotic functions, including the activation of autophagy (Kuranaga 2012; Denton et al, 2012a). In insects, autophagy occurs in tissues undergoing degradation during morphogenesis (Berry & Baehrecke, 2007; Lee et al, 2002; Rusten et al, 2004), where autophagic vesicles have been associated with type II programmed cell death, or autophagic cell death (Kroemer & Levine, 2008). Genetic screens in *D. melanogaster* show that pro-apoptotic proteins positively regulate autophagy, while IAPs act as negative regulators (Hou et al, 2008; Kim et al, 2010). Our data indicate that *Aedronc*, *Casps7* and several other apoptosis-related genes are transcriptionally active during programmed autophagy in *Ae. aegypti*, including *Casps7*, *Aep53* and several IAP orthologs (*IAP5, IAP9, Aebruce*). This complements and confirms other transcription studies where autophagic and IAP genes were upregulated in other strains of *Ae. aegypti* following blood feeding (Bonizzonni et al, 2011; Bonizzoni et al, 2012b; Puglise et al, 2015). In this study, we present a model where caspases are involved with the control of programmed autophagy post meal meal (Fig 9B). Initiator caspase *Ae*dronc controls transcription or stability of effector caspase Casps7. Casps7 can promote either apoptosis or autophagy, possibly with the guidance of other apoptosis-related proteins. The mechanism by which Casps7 could activate autophagy is still unknown. In *D. melanogaster*, a Casps7 ortholog promotes autophagy by inhibiting translocation of the autophagy inhibitor, ATP, during oogenesis (DeVorkin et al, 2014). Differences in activity of the pro- and cleaved forms of effector caspase may promote autophagy without inducing apoptosis. Interestingly, Kim et al (2010) found that in *D. melanogaster* full length Casps7 promoted autophagy, whereas expression of the truncated version induced apoptosis. Thus, the subactivation of caspases can lead to substrate cleavage without the induction of apoptosis.

In summary, we present evidence that the initiator caspase *Ae*dronc is important for DENV infection in *Ae. aegypti*, possibly through its regulation of autophagy. Autophagy has previously been implicated in the replication and infection of DENV in mammalian cells. Inhibition of autophagy in murine systems can reduce DENV infection by over 1000-fold (Lee et al, 2008; Heaton & Randall, 2010; McLean et al, 2011; Mateo et al, 2013). In addition, stimulation of autophagy in epithelial cells by DENV NS4A protein protects them against DENV-induced apoptosis (McLean et al, 2011). Autophagy also promotes DENV infection by altering lipid metabolism and enhancing maturation of infectious particles (Heaton & Randall, 2010; Panyasrivit et al, 2009; Mateo et al, 2013), and it is possible that cellular components freed by autophagy could be important for the rearrangement of internal membrane structures that act as sites of replication (Junjhon et al, 2014). While these DENV-autophagy connections have not yet been tested in *Ae. aegypti*, we previously found transcriptional upregulation of autophagy genes *ATG4A*, *Aedebcl* and *Casps7* in MS strain and not MR strain following DENV infection (Behura, S.K. & Severson, D.W., unpublished data; Chauhan et al, 2012). Given the importance that programmed autophagy plays in mosquito physiology following vitellogenesis and the infection of murine hosts, it is probable that this process is important in infection of vector hosts as well, but has yet to be determined. Understanding such host factors is necessary in order to develop novel methods of blocking transmission of vector-borne diseases.

Highlights

- DENV-refractory, but not susceptible mosquitoes show increased apoptosis of midgut tissue upon DENV-2 infection.
- Unexpectedly, silencing the initiator caspase *Aedronc* decreases DENV-2 dissemination and infectious titer in *Aedes aegypti*.
- Programmed autophagy is activated following a blood meal, and several apoptosis-related genes show upregulation paralleling this process.
- Knockdown of *Aedronc* and the effector caspase, *Casps7,* reduces programmed autophagy in midgut tissues.

Supplementary Material

1

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2

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3

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4

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5

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Acknowledgements

Abbreviations

DENV	dengue virus
SINV	Sindbis virus
MS	Moyo-S
MR	Moyo-R
IAP	Inhibitor of apoptosis
Mx	Michelob_x
ATG	autophagy-related protein
HPI	hours post injection
PBM	post blood meal
RH	relative humidity
DENV	dengue virus
MOI	multiplicity of infection
qPCR	quantitative PCR
RPS17	ribosomal protein S17
RPL8	Ribosomal protein L8
B-gal	B-galactosidase
HCQ	hydroxychloroquine
FBS	fetal bovine serum
PE	Phosphatidyl ethanolamine

RQ   relative quantitation

Footnotes

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