Aedes Dronc: a novel ecdysone-inducible caspase in the yellow fever mosquito, Aedes aegypti

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Abstract

Caspases are cysteinyl-aspartate-specific proteases known for their role in apoptosis. Here, we describe the characterization of Aedes Dronc, a novel caspase in the yellow fever mosquito, Aedes aegypti. Aedes Dronc is predicted to contain an N-terminal caspase recruitment domain and is a homologue of Drosophila Dronc and human caspase-9. An increase in transcripts and caspase activity coincides with developmental changes in the mosquito, suggesting that Aedes Dronc plays a role in developmental apoptosis. Exposure of third instar larvae to ecdysone resulted in a significant increase in both transcript levels and caspase activity. We present here a functional characterization of the first caspase recruitment domain-containing caspase in mosquitoes, and will initiate studies on the role of apoptosis in the innate immune response of vectors.

Keywords: apoptosis, caspase, Aedes aegypti, ecdysone, development.

Introduction

Programmed cell death, in particular apoptosis, is an essential component of metazoan development, and contributes towards tissue homeostasis and immunity (Hengartner, 2000; Kaufmann & Hengartner, 2001; Lawen, 2003). The regulation and control of apoptotic cell death is a highly conserved process culminating in the activation of a family of cysteine-specific proteases called caspases. Caspases are expressed aszymogens and contain an N-terminal prodomain, and a C14 peptidase domain containing a large (17–20 kDa) and small subunit (10–12 kDa) (Teodoro & Branton, 1997; Raff, 1998; Thornberry & Lazebnik, 1998; Sehra & Dent, 2006). Caspases are activated by proteolytic cleavage at sites located between these domains that removes the prodomain and releases the large and small subunits. Apoptotic caspases are divided into two classes (Sehra & Dent, 2006). The initiator caspases (human caspases-8, -9,-10) are characterized by the presence of long N-terminal prodomains that contain protein–protein interaction motifs such as caspase recruitment domains (CARDs) or a pair of death effector domains (DEDs) (Nicholson, 1999; Ho & Hawkins, 2005). These protein interaction motifs are used to recruit initiator caspases to multi-protein complexes that result in their auto-activation. Once activated, the initiator caspases cleave and activate the second class of caspases, the effector caspases (human caspases-3, -6, -7), characterized by the presence of short N-terminal prodomains. Effector caspases execute the cell death process by cleaving a large number of cellular proteins that bring about the dismantling of the cell.

The Drosophila genome contains seven caspase genes (Kumar & Doumanis, 2000; Richardson & Kumar, 2002). Of these, Dronc, Dredd and Strica are predicted to be initiator caspases, whereas the remaining four, Drice, Dcp-1, Damm and Decay are predicted to be effector caspases. Dronc and Dredd carry N-terminal CARD and DED domains respectively, while Strica contains a large N-terminal prodomain but lacks any known death domain motifs (Chen et al., 1998; Dorstyn et al., 1999; Adrain & Martin, 2001). Dronc is the only known Drosophila caspase that carries a CARD motif in its prodomain and is functionally most similar to human caspase-9.

The function of individual caspases in different cell death pathways is just beginning to be understood. Drosophila caspases function in both immune signalling pathways and in insect development. Insect metamorphosis is a complex, strictly regulated process/progression of developmental events that is triggered by a number of signals, including...
the steroid hormone ecdysone. During metamorphosis some tissues, such as the salivary glands and gut, are completely histolysed, while other predetermined imaginal tissues differentiate and grow into organs and external structures (Lee et al., 2002). A number of Drosophila apoptotic proteins are involved in developmental cell death, including Dronc, Reaper and Hid. Drosophila Dronc is ubiquitously expressed throughout development and is a known target of ecdysone; an elevation in dronc transcripts correlates with ecdysone peaks and the degradation of the larval midgut and salivary gland tissues (Cakouros et al., 2004). All dronc mutant alleles are early pupal lethal when homozygous, demonstrating the essential role for dronc during development (Daish et al., 2004; Chew et al., 2004; Xu et al., 2005; Waldhuber et al., 2005; Kondo et al., 2006).

Little is known about apoptosis in mosquitoes, although apoptotic-like cell death has been reported previously in the midgut cells and ovarian follicles of Plasmodium-infected Anopheles gambiae (Zieler & Dvorak, 2000; Al-Olayan et al., 2002; Hurd et al., 2006). While this activity was shown to be caspase-like, the caspses involved in this process were not characterized (Al-Olayan et al., 2002; Hurd et al., 2006). We present here the identification and characterization of Aedes Dronc, the first CARD domain-carrying caspase in the yellow fever mosquito, Aedes aegypti. Aedes Dronc is a homologue of Drosophila Dronc and human caspases-2 and -9. Aedes Dronc contains the well conserved C14 peptidase subunit with a unique sequence, SICRG, surrounding the catalytic Cys. Recombinant Aedes Dronc exhibits caspase activity on substrates specific for human caspases-9 and -2. Aedes Dronc also contains a putative N-terminal CARD domain, suggesting that an intrinsic apoptotic pathway exists in mosquitoes. As is the case in Drosophila, Aedes Dronc appears to be involved principally in insect development with the highest levels of transcripts and caspase activity detected in late instar larvae and early and late pupae, which corresponds to tissue reorganization and pulses of the steroid hormone ecdysone.

Results
Identification of Aedes Dronc
We performed a TBlastN search of the TIGR Aedes genomic database using protein sequence queries of both the C14 peptidase subunits and the prodomains of Drosophila and mammalian caspases and identified a draft sequence that shared 36% identity with Drosophila Dronc. Through 5′–3′ RACE PCR we obtained a cDNA with an open reading frame that encodes a novel 449 residue caspase named Aedes Dronc. Aedes Dronc shares 99% identity with a putative uncharacterized protein present in the NCBI database (GenBank accession: gi:78216591). Phylogenetic analysis clusters Aedes Dronc with Drosophila Dronc and human caspases-1, -2 and -9 (Fig. 1). Comparisons with the conserved domain database indicate that Aedes Dronc contains a C-terminal C14 peptidase subunit [251 amino acids (aa)] and an N-terminal prodomain 198 aa in length. Multiple sequence alignments with mammalian and invertebrate caspases indicate that Aedes Dronc contains all the essential residues required for catalysis (His-237, Gly-238 and Cys-285) and the residues involved in coordinating the P1 aspartate (Arg-179, Glu-283, Arg-341, Ser-343), using caspase-1 nomenclature (Fig. 2A) (Earnshaw et al., 1999). The C-terminus of Aedes Dronc shares significant sequence identity with the C14 peptidase subunits of all mammalian and insect caspases, with the highest similarity with Drosophila Dronc (36% identity, 55% similarity). Aedes Dronc shares 29% identity (49% similarity) with the C14 peptidase subunit of human caspase-2; 29% identity (49% similarity) with human caspase-9 (Fig. 2A). The sequence encompassing the catalytic Cys in Aedes Dronc is unique and contains SICRG.

Although caspase prodomains tend to be much more divergent than the catalytic segments, we identified limited sequence similarities among the prodomains of Aedes Dronc, Drosophila Dronc, human caspase-2 and human caspase-9. The prodomain of Aedes Dronc shares the greatest identity with the prodomain of Drosophila Dronc (31% identity; 54% similarity), and limited sequence identity with the prodomains of human caspase-2 (10% identity; 20% similarity) and human caspase-9 (10% identity; 19% similarity). We used a fold recognition program (BiofoBank Metaserver; http://meta.bioinfo.pl) to predict secondary structure in the prodomain of Aedes Dronc. The program predicts one six-α-helix bundle in the prodomain of Aedes Dronc, nearly identical to that found in Drosophila Dronc and similar to those found in human caspases-2 and -9 (Fig. 2B).

Expression during development
Quantitative real-time PCR (qPCR) detected Aedes dronc transcripts in all developmental stages. Transcript levels in third instar larvae were 26-fold higher than levels in the early instar stages. Transcript levels in fourth instar larvae were 12-fold higher than those in the third instar larvae, while transcript levels in callow and black pupae were 126 and 53-fold higher, respectively, than third instar larvae (Fig. 3A).

Tissue-specific expression
Aedes dronc transcripts were found in differing levels in all individual adult tissues screened in this study. Transcript levels were highest in the fat body and gut tissues; six- to eight-fold higher than the levels found in salivary glands and approximately two-fold higher than ovary tissues (Fig. 3B).
Enzymatic activity and substrate specificity of recombinant Aedes Dronc

We expressed and incubated the C14 peptidase subunit of Aedes Dronc with a variety of caspase-specific colorimetric tetrapeptide substrates to analyse substrate preference. Aedes Dronc displayed significant cleavage on VDVAD-pNA (optimal for caspase-2) and LEHD-pNA (optimal for caspase-9) (Fig. 4A). Minimal activity was also detected on AEVD-pNA (optimal for caspase-10) and WEHD-pNA (optimal for caspase-5). The activity of Aedes Dronc on VDVAD-pNA was suppressed by all concentrations of the general caspase family inhibitor z-VAD-fmk (Fig. 4B).

Caspase activity during development

We assessed caspase activity during different developmental stages of Ae. aegypti by mixing total cell protein extracted from early instar, late instar, callow and black pupae with the caspase-3 substrate DEVD-pNA and the caspase-9 substrate LEHD-pNA. Although recombinant Aedes Dronc was shown to prefer VDVAD-pNA, it was not used in this developmental assay because previous work in our lab indicates that other mosquito caspases also use VDVAD-pNA (data not shown). Consistent with our expression data, cell extracts prepared from late instar larvae, early (callow) and late (black) pupae showed the highest activity on the caspase-9 substrate, suggesting that the increase in Aedes dronc transcripts correlates with active Aedes Dronc protein. In addition, subsequent caspase-3-like activity was detected in all developmental stages, suggesting involvement of downstream effector caspases (Fig. 5). Caspase activity in the developmental assays was greatly reduced by the addition of 2 μM z-VAD-fmk, confirming that the activity we were measuring was caspase specific.

Ecdysone treatment

We examined transcript levels of Aedes dronc in third instar larvae after a 24 or 48 h exposure to ecdysone. We detected a six-fold increase at 24 h and a 16-fold increase in Aedes dronc transcripts after 48 h of exposure (Fig. 6A). An analysis of total cell protein from larvae treated with ecdysone showed an increase in both Aedes Dronc specific activity (LEHD-pNA) and caspase-3-like activity (DEVD-pNA). LEHD-pNA activity increased to 24 h and remained relatively constant to 48 h, whereas the activity on DEVD-pNA steadily increased to 48 h (Fig. 6B).
Discussion

Caspases are a family of cysteine proteases responsible for the concerted and coordinated action of apoptosis. Caspases are expressed as pro-enzymes composed of three domains: an N-terminal prodomain and a large and small catalytic subunit. Activation requires proteolytic cleavage among these domains and the formation of heterodimers.
by the large and small subunits (Thornberry & Lazebnik, 1998). These inter-domain cleavage sites are themselves caspase consensus sites, indicating that caspases function as part of a proteolytic cascade (Cohen, 1997) that involves the auto- and trans-activation of apical caspases which then cleave and activate downstream effector caspases (Nicholson, 1999). Apoptotic caspases are classified as initiators which contain long prodomains, and effectors which contain short prodomains.

The large prodomains of initiator caspases contain CARD (human caspases-1, -2, -4, -5, -9) and DED (human caspase-8 and -10) domains that are used to recruit the initiator caspases to multi-protein complexes for auto-activation (Nicholson, 1999; Ho & Hawkins, 2005). Four initiator caspases have been identified in insects: Drosophila Dronc, which carries a CARD domain; Drosophila Dredd, which carries two DEDs; Drosophila Strica, which carries a...
serine/threonine rich region; and Aedes Dredd which carries two DEDs (Chen et al., 1998; Dorstyn et al., 1999; Kumar & Doumanis, 2000; Adrain & Martin, 2001; Cooper et al., 2007). CARD and DED are homotypic domains required for the transmission and regulation of signals from death receptors to apoptotic effector molecules, such as the caspases (Tibbetts et al., 2003). In general, these homotypic interaction domains share a low degree of sequence similarity but share a common three-dimensional fold, classified as the death domain-fold in SCOP (Structural Classification of Proteins) (Murzin et al., 1995). Even within the death domain family, the sequences can be so divergent that statistically significant sequence similarities cannot be detected by conventional sequence comparison. The death domain-fold comprises an antiparallel six-helix bundle. By sequence comparison, the prodomain of Aedes Dronc shares 31% sequence identity with the prodomain of Drosophila Dronc, 10% identity with the prodomain of human caspase-2 and 10% identity with the prodomain of caspase-9. Despite the lack of obvious sequence similarity, the prodomain of Aedes Dronc is predicted to contain one six-α-helix bundle, nearly identical to that seen in Drosophila Dronc (Fig. 2B), indicating that Aedes Dronc is a CARD-carrying caspase.

In addition to N-terminal prodomains, caspases contain a conserved C14 peptidase domain, comprising a large (17–20 kDa) and small (10–12 kDa) subunit. The catalytic domain of Aedes Dronc shares a high degree of sequence similarity with the catalytic domains of many known caspases, with the highest similarity found with Drosophila Dronc. Aedes Dronc contains all the residues involved in caspase catalysis; however, the sequence encompassing the catalytic Cys residue in Aedes Dronc is SICRG, different from the QAC(R/Q/G)(G/E) found in most known caspases. Drosophila Dronc also has a unique catalytic sequence, PFCRG, surrounding the catalytic Cys (Fig. 2A), which accounts for its unique specificity (Dorstyn et al., 1999). Aedes Dronc showed significant activity on VDVAD-pNA and LEHD-pNA and minimal activity on WEHD-pNA and AEVD-pNA (Fig. 4A). LEHD-pNA is a substrate preferred by human caspase-9 while VDVAD-pNA is the optimal substrate for caspase-2. This differs from the substrate specificity observed with Drosophila Dronc, which also shows activity on VEID, IETD and VDVA substrates, and minimal activity on DEVD-containing substrates (Dorstyn et al., 1999; Hawkins et al., 2000).

In general, caspases show a high degree of specificity, and cleave after an aspartic acid (Talanian et al., 1997). Drosophila Dronc is unique among caspases because it will cleave after an aspartate residue (similar to other caspases) but also will cleave after glutamate residues (Hawkins et al., 2000; Muro et al., 2004; Hay & Guo, 2006). Dronc activation involves cleavage at E352, triggering the transition from an inactive monomer to a catalytically active dimer (Hay & Guo, 2006). Following cleavage at E352, Dronc undergoes autocleavage at E143 (Muro et al., 2004; Yan et al., 2006), releasing the CARD domain from the active catalytic dimer.

Sequence analysis indicates that both E143 and E352 are conserved in Aedes Dronc (Fig. 2B), suggesting that Aedes Dronc activation proceeds through a similar mechanism. The specific activation and ability of Aedes Dronc to cleave after a glutamate will be the subject of future studies. Cleavage after E143 in Drosophila Dronc removes the DIAP1 binding site. DIAP1 belongs to the IAP (Inhibitors of Apoptosis Proteins) family of proteins which can inhibit caspase function by binding to the active caspase site, by promoting degradation of active caspases or by sequestering the caspases away from their substrates (Tenev et al., 2005). DIAP1 contains a BIR domain that binds to a 12-residue...
sequence in Dronc (114-SRPPFILNFD-125) located between the CARD domain and the large catalytic subunit (Chai et al., 2003). The C-terminal RING finger domain of DIAP1 then targets Dronc for ubiquitination (Wilson et al., 2002; Chai et al., 2003). There is conservation, albeit limited, of the 12-residue DIAP1 binding site in Aedes Dronc, suggesting that Aedes Dronc activity may also be regulated by a DIAP1 homologue in mosquitoes (Fig. 2B). Two IAP molecules, AtIAP and AaIAP1, have been identified in mosquitoes (Blitvich et al., 2002; Li et al., 2007). AaIAP1 appears to be the mosquito homologue of DIAP1 (Li et al., 2007) and investigation of the ability of AaIAP1 to interact with and inhibit Aedes Dronc activity is currently underway.

Aedes dronc transcripts were found in all adult tissues screened in this study, with the highest levels found in the fat body, a tissue with high cell turnover rates and the primary immune response tissue in insects (Fig. 3B). Aedes dronc was ubiquitously expressed during mosquito development, with the highest levels of transcript found in the pupal stages (Fig. 3A). In insects, the steroid hormone ecdysone regulates cell death and differentiation during development. An ecdysone pulse toward the end of third larval instar development in Drosophila induces imaginal disc evagination, a dramatic change in salivary gland gene transcription and larval midgut cell death (Baehrecke, 2000; Jiang et al., 2000; Lee et al., 2002). A second pulse of ecdysone in the early pupal stage then triggers salivary gland cell death. Studies have shown that dronc and its effector molecules, rpr and hid, are barely detectable in early instar larvae but are transcriptionally upregulated in response to the pulses of ecdysone in late third instar larvae prior to developmental apoptosis (Jiang et al., 2000; Daish et al., 2003; Cakouros et al., 2004). Metamorphosis in mosquitoes is similarly triggered by pulses of ecdysone. In the presence of juvenile hormone, ecdysone triggers larval–larval moults (Margam et al., 2006). During the late fourth instar, a small peak of ecdysone, in the absence of juvenile hormone, is responsible for larval commitment to pupal development. A second peak of ecdysone triggers apoptosis, followed by formation of the pupal cuticle. In the pupal stage, a large peak of ecdysone triggers apoptosis and the initiation of adult tissue formation (Clements, 1992; Margam et al., 2006). The increase in Aedes dronc transcripts observed in the fourth instar larvae and the early (callow) and late (black) pupae stages is correlated with both LEHD-pNA activity, which suggests Aedes Dronc activity, and with DEVD-pNA which suggests downstream effector caspase activity. Although we do not have a complete substrate usage profile for all of the caspases that exist in mosquitoes, the profile determined for Aedes Dronc, in conjunction with the strong upregulation of Aedes dronc transcripts, suggests that Aedes dronc may also be developmentally regulated and involved in the apoptotic destruction of larval tissues during metamorphosis (Fig. 5).

An increase was detected in dronc transcripts in the midgut tissues of fourth instar Aedes aegypti larvae but not in the gut tissues of the early pupal stage (Wu et al., 2006). We detected an increase in dronc transcripts in the whole bodies of both fourth instar larvae and pupal stages. The additional peak of Aedes dronc transcripts in the pupal stages may represent the apoptotic destruction of other tissues such as the salivary glands (Daish et al., 2003; Cakouros et al., 2004). To investigate whether ecdysone induces the expression of Aedes dronc in mosquitoes, we examined the levels of Aedes dronc transcripts in third instar larvae following treatment with ecdysone. Third instar larvae were chosen because they normally only show low levels of both dronc transcripts and caspase activity. We detected a 16-fold increase in dronc transcripts and a large increase in both LEHD-pNA and DEVD-pNA caspase activity in larvae 48 h after ecdysone treatment. The activity on LEHD-pNA increased at 24 h and remained relatively constant to 48 h, whereas the activity on DEVD-pNA steadily increased to 48 h. This pattern of activation suggests that Aedes Dronc is an initiator caspase responsible for the activation of downstream effector caspases. These data, together with recent studies, suggest that many caspases, including Aedes Dronc, mediate tissue and stage-specific apoptosis during insect development (Mills et al., 2005).

Few studies have addressed the role of apoptosis in insects that transmit human parasites. The identification of Aedes Dronc as a CARD-containing caspase, and as a homologue of Drosophila Dronc, suggests that a Dronc/caspase-9 apoptotic pathway may also exist in mosquitoes. In addition to development, apoptosis is a known mediator of the immune response (Schaumburg et al., 2006). Apoptotic-like activity has been associated with Plasmodium infection in Anopheles gambiae, suggesting a role for apoptosis in the mosquito immune response (Zieler & Dvorak, 2000; Hopwood et al., 2001; Al-Olayan et al., 2002; Hurd et al., 2006). The identification of a caspase central to a Drosophila Dronc/caspase-9 pathway will provide a stepping stone to characterizing the role that apoptosis and caspases play in mosquito immunity. The role of Aedes Dronc in the immune response of Aedes aegypti will be the subject of further study. Information on the effector molecules that regulate the apoptotic response, such as caspases, will provide insight into the mechanisms that insect vectors use to regulate intracellular infections and provide a means to examine the evolution of apoptotic pathways as they relate to development and immunity.

**Experimental procedures**

*Insect maintenance, cDNA generation*

*Aedes aegypti* (Liverpool strain) mosquitoes were reared as described previously (Lowenberger et al., 1999). Total RNA was
extracted from different developmental stages (larvae, callow pupae, black pupae and whole adult mosquitoes) or from individual tissues (salivary glands, ovaries, midguts and fat body tissues) as described previously (Cooper et al., 2007). RNA concentration was determined using a spectrophotometer (Eppendorf, Hamburg, Germany) and reverse transcription was performed using 3 μg of total RNA. All reverse transcription reactions were performed as described previously (Lowenberger et al., 1999), with the modification of using a 40 °C incubation temperature for 60 min. All cDNAs were screened with primers known to span an intron to verify that samples contained no genomic DNA contamination.

Identification and sequencing Aedes Dronc
Partial sequences of Aedes Dronc were identified in a series of TBLASTN searches of the TIGR Aedes Gene Indices (http://www.tigr.org/tbd/tgi/) as an expressed sequence tag (EST) displaying significant homology to the C14 peptidase subunit of Dro sophila and mammalian caspases. Primers designed against the EST sequences were used to obtain partial cDNA segments from our whole body and tissue-specific cDNAs. 5′−3′ RACE PCR (Marathon cDNA Amplification kit; Clontech, Mountain View, CA, USA) was used to obtain the full length cDNA. Purified PCR products were cloned into pGEM T-Easy (Promega, Madison, WI, USA) and sequenced using Big Dye v3.1 chemistry (ABI, Foster City, CA, USA). Sequence information was used to design primers for qPCR studies.

Tissue-specific and developmental comparisons
For an estimation of tissue-specific expression and mRNA concentration we used qPCR. All qPCR reactions were performed with a Rotor-Gene 3000 (Corbett Research, Mortlake, NSW, Australia) using the Platinum SYBR Green Supermix-UDG (Invitrogen, Carlsbad, CA, USA). We used 2.5 μl cDNA with 12.5 μl of Platinum SYBR Green Supermix, 1 μl (10 μM) sense primer (5′-CGAACGGACAGAGACACGTTT-3′), 1 μl (10 μM) antisense primer (5′-GGGACAGATTGAGAAAAAGAAC-3′) in 25 μl reactions under the following conditions: 50 °C for 2 min, 95 °C for 2 min, followed by 35 cycles of 95 °C for 10 s, 61.5 °C for 15 s, 72 °C for 30 s. Quantity values were generated using the 2−ΔΔCt method as described previously (Livak & Schmittgen, 2001). The expression of Aedes dronc was compared per unit of β-actin, a normalizing gene that had stable expression levels in all conditions tested. All data represent triplicate runs of independently generated cDNAs.

Expression of recombinant Aedes Dronc, caspase assays, and inhibition assays
Recombinant Aedes Dronc was generated by transforming Escherichia coli BL21 (E3) cells with a truncated form of Aedes Dronc lacking the putative prodomain (residues 1−198) (Fig. 1A). The C14 subunit was amplified by PCR and cloned into pET-46 EK/LIC (Novagen, Madison, WI, USA). pET-Aedes Dronc LB-Amp85 starter cultures were grown for 3 h, subcultured into fresh medium and grown at 37 °C to an Optical Density (OD) of 0.5. Cultures were induced with 2 mM IPTG and grown for 16 h at 22 °C. Cells were pelleted and lysed using Easy-Lyse Bacterial Protein Extraction Solution (Epicentre, Madison, WI, USA) following the manufacturer’s instructions. Cleared E. coli lysates from cells expressing Aedes Dronc were assayed for protein concentration using the Bradford Assay (BioRad, Hercules, CA, USA), and 100 μg of total protein from each lysate was incubated with 200 μM chromophore p-nitroaniline-labelle substrates for 3 h at 37 °C according to the manufacturer’s recommendations (BioVision Inc., Mountain View, CA, USA). The release of pNA was monitored by a spectrophotometer at 405 nm. Activity was considered biologically significant when absorbance was two-fold higher than the uninduced controls (Biovision Inc.). We used the general caspase inhibitor, z-VAD-fmk (Biovision Inc.), to inhibit caspase activity by interfering with the processing of the substrate VDVAD-pNA. For each assay, a given amount of z-VAD-fmk was mixed with 100 μg of Aedes Dronc lysate (total protein) and allowed to incubate for 1 min. 200 μM VDVAD-pNA was added and each reaction was allowed to incubate for 3 h at 37 °C. The data represent three replicates, measured in duplicate.

Caspase activity in developmental stages
To determine if changes in gene expression were correlated with caspase activity, we measured caspase-9 and caspase-3 activity in cell extracts prepared from early instar, late instar and callow and black pupae. Larvae or pupae were ground in 1× Phosphate Buffered Saline (PBS) and frozen at −80 °C. Cells were fractured with four freeze−thaw cycles (−80 °C for 5 min and fast thawed for 30 s at 42 °C). Insect carcasses were removed by centrifugation at 10 000 g for 1.5 min. Cells were lysed with 200 μl cell lysis solution (Biovision Inc.). Phenylthiourea (15 mM final concentration) was added to each cell extract to prevent melanization. Total cell protein was measured as described above and 100 μg total cell protein from each cell lysate was incubated with 200 μM DEVD-pNA or LEHD-pNA for 3 h at 37 °C according to the manufacturers’ recommendations. The release of pNA was monitored by a spectrophotometer at 405 nm. To ensure the activity we were detecting in these assays was the result of caspase specific activity, we added 2 μM z-VAD-fmk to 100 μg total cell protein as described above.

Ecdysone treatments
Early third instar larvae were rinsed with fresh ddH2O and maintained individually for 3 h in tissue culture wells containing 100 μl of water and 30 μM 2-hydroxycycadsone (ecdysone) (Sigma, Oakville, ON, Canada) for 24 and 48 h at 22−25 °C. Water volumes were increased to 1 ml/well and finely ground Tetramin® (Montreal, QC, Canada) fish flakes were added as a food source 3 h after initial ecdysone exposure. At specific time points, whole larvae were placed directly in Tri-Reagent and RNA extraction, cDNA synthesis and qPCR were performed as described above. Larvae treated with water served as the control. Caspase activity at 0, 24 and 48 h after ecdysone treatment was assessed using 200 μM LEHD-pNA or DEVD-pNA and was conducted as described above.

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