Molecular characterization and expression analysis of three novel autophagy-related genes from the cattle tick *Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodidae)

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SUMMARY

The cattle tick *Rhipicephalus* (*Boophilus*) *microplus* is a hematophagous ectoparasite of major importance for the livestock industry. It shows a remarkable ability to survive over long periods without feeding. However, the mechanisms used to endure long-term starvation are poorly understood. It is believed that autophagy, a process of intracellular protein degradation, may play a significant role to confront adverse environmental conditions. To advance our understanding of autophagy in *R. microplus*, in the present study we report the molecular characterization of three autophagy-related (*ATG*) genes, namely, *RmATG3*, *RmATG4* and *RmATG6*, as well as their expression profiles in different developmental stages and organs of the parasite. The deduced amino acid sequences derived from the characterized gene sequences were subjected to Basic Local Alignment Search Tool analysis. The testing produced significant alignments with respective ATG proteins from *Haemaphysalis longicornis* and *Ixodes scapularis* ticks. Real-time polymerase chain reaction assays revealed that *RmATG4* and *RmATG6* transcripts were elevated in egg and ovary tissue, when compared with larva and midgut samples, while *RmATG3* expression in midgut was 2-fold higher than in egg, larva and ovary samples.

Key words: Autophagy, ATG3, ATG4, ATG6, *Rhipicephalus microplus*, real-time PCR, development, embryogenesis.

INTRODUCTION

The cattle tick *Rhipicephalus* (*Boophilus*) *microplus* is a hematophagous ectoparasite of major importance for the livestock industry. Feeding by large numbers of ticks causes reduction in live weight gain and milk production as well as deterioration of the skin on parasitized cattle. Further, *R. microplus* transmits diseases of economic importance such as babesiosis and anaplasmosis (Sharma *et al.* 2012). Its remarkable ability to survive without feeding over long periods makes its control and eradication difficult.

Under optimal environmental conditions *R. microplus* is able to complete its life cycle in 3–4 weeks; however, in cooler temperatures larvae may survive without feeding for up to 24 weeks (Spickler, 2007). The mechanisms used to survive long-term starvation are poorly understood in ticks. However, it is believed that autophagy may play a significant role, as a strategy to confront adverse environmental conditions (Umemiya-Shirafuji *et al.* 2010).

Macroautophagy (hereafter referred to as autophagy) is a cytoplasmic degradation process where organelles and proteins undergo proteolysis to obtain polypeptides in order to maintain amino acid pools and energy balance in the cell. Autophagy is regulated by a set of evolutionarily conserved gene products, known as the ATG proteins (Mizushima *et al.* 2010). Among *ATG* genes one subset, composed of 17 genes, is required for efficient autophagosome formation. The corresponding gene products are referred to as the core machinery for autophagosome formation (Xie and Klionsky, 2007).

In a previous work, we reported the first molecular characterization of two autophagy-related genes, *RmATG8a* and *RmATG8b*, homologues of mammalian *GABARAP* and *MAP1LC3*, respectively, in *R. microplus*. We found that transcripts of both *RmATG8a* and *RmATG8b* were up-regulated during starvation, suggesting that autophagy was active in *R. microplus* (Flores *et al.* 2014). To advance our understanding of autophagy in this tick, in the present study we report the molecular characterization of *RmATG3*,

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RmATG4 and *RmATG6* as well as their expression profiles in different developmental stages and organs of the parasite. The products of all of these genes are part of the core machinery for the autophagosome formation.

The autophagy process seen as a survival strategy of ticks could also be important for the survival of pathogens they transmit. Therefore, targeting autophagy mediators could lead to the development of novel control strategies for ticks and associated pathogens.

MATERIALS AND METHODS

Ticks and tissues

Rhipicephalus microplus ticks were obtained from a susceptible strain reared under laboratory conditions at CENID-PAVET-INIFAP, located in the State of Morelos, Mexico. Ovarian and intestinal tissues were obtained from engorged female ticks the following day after the spontaneous detachment from calves, as described by Tsuda *et al*. (2001). Some engorged female ticks were maintained at 28 °C and 80% relative humidity (RH) to collect eggs 8 days after oviposition and larvae 8 days after hatching.

Treatment of eggs

100 mg of eggs were treated with 5 volumes of 3·8% NaClO for 2 min and centrifuged at 370 ***g*** for 2 min. The supernatant was removed and eggs were resuspended in 5 volumes of 3·8% NaClO and incubated for 10 min. Floating eggs were removed and the remaining eggs were rinsed 5 times with 5 volumes of distilled water.

RNA isolation and cDNA synthesis

Total RNA was extracted from eggs, larvae and adult tissues using RNeasy Mini Kit (Qiagen, Germany). Briefly, midgut and ovary tissues were dissected and pooled from 15–20 engorged females; tissues were maintained on ice-cold phosphate-buffered saline immediately after dissection and then stored at −80 °C until RNA extraction. Pools of 100 mg eggs (approximately 2000 eggs) and larvae (approximately 1000 larvae) were collected. Eggs were treated as mentioned previously before RNA extraction. Samples were mixed and crushed with liquid nitrogen. RNA integrity was assessed by denaturing electrophoresis on 1% agarose gels and quantified by a ND-1000 NanoDrop spectrophotometer (NanoDrop Products, Wilmington, DE, USA). cDNA was synthesized from 2 μg of RNA using the SuperScript III® First-Strand Synthesis System (Invitrogen) according to manufacturer's instructions.

Characterization of the complete coding sequences

To obtain the complete coding sequence of *RmATG3, RmATG4* and *RmATG6* we started from the predicted putative sequences reported by Flores-Fernández *et al*. (2014). Briefly, the nucleotide sequences of *HlATG3, HlATG4* and *HlATG6* genes from *Haemaphysalis longicornis* tick (GenBank accession numbers AB513349, AB513350 and AB601889, respectively) were queried against the GenBank *R. microplus* Expressed Sequence Tags (EST) database, using the Basic Local Alignment Search Tool (BLAST) tool. We retrieved the nucleotide sequences CK182322 (EST771642) and CV442344 (EST896257) for *ATG3*; CK181180 (EST770500) and CK181181 (EST770501) for *ATG4*; CV442171 (EST896084) and CV442170 (EST896083) for *ATG6* and proceeded with their characterization. Adopting the nomenclature followed by Umemiya-Shirafuji *et al*. (2010) to name tick *ATG* genes, we designated the characterized genes as *RmATG3, RmATG4* and *RmATG6*, respectively. Specific primers were designed to the above sequences (online Table S1). The forward RmATG4 primer was designed from a conserved region of the sequence of *HlATG4* of *H. longicornis* and two Expressed Sequence Tag (EST) sequences of *Ixodes scapularis* and *Amblyomma americanum* (GenBank accession no. AB513350, EW946975 and JZ176775, respectively). End-point polymerase chain reaction (PCR) reactions were performed using cDNA synthesized from larvae as a template. PCR conditions were 94 °C for 5 min followed by 35 cycles, each consisting of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 60 s. A final extension step at 72 °C for 10 min was included. The amplified products were visualized on 1% agarose gels on a UV transilluminator (Bio-Rad Laboratories, Philadelphia, PA, USA).

Cloning and sequencing

To confirm positive PCR products, amplicons were purified and then cloned into the pCR®2.1-TOPO T/A plasmid vector (Invitrogen, Carlsbad, CA, USA) which was used to transform electro-competent *Escherichia coli* TOP10 cells. *Escherichia coli* cells were then incubated overnight at 37 °C on LB plates containing ampicillin (50 μg mL^{-1}) and 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal; 40 mg mL^{-1}). Five white colonies were isolated and cultured overnight in LB medium containing 50 µg mL^{-1} ampicillin. Plasmid DNA was extracted using QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. To confirm the positive transformants the plasmids were analyzed by PCR using forward and reverse M13 primers. Once the correct clones were identified, the plasmids were submitted for sequencing at Macrogen Corp. (Rockville, Maryland, USA). The new sequences of *RmATG3, RmATG4* and *RmATG6* were deposited in GenBank with accession number KP317124, KR822806 and KR822807, respectively.

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Gene expression by quantitative PCR

To determine the expression profiles of *RmATG3, RmATG4* and *RmATG6* genes in eggs, larvae and adult tissues of *R. microplus*, specific primers were designed (online Table S1). *Elongation factor 1-α* (*ELFIA*) and *β-actin* (*ACTB*) genes were used as reference genes (Nijhof *et al.* 2009). Real-time PCR amplifications were performed in 96-well-plates using SYBR Green detection chemistry in a StepOnePlus™ Real-time PCR System (Applied Biosystems/Ambion, Austin, TX, USA). Reactions were prepared in a total volume of 15 μL containing 0·4 μL of first strand cDNA template (100 ng μL^{-1}), 0·45 μL of forward and reverse primers (10 μM), 7·5 μL of SYBR® Select Master Mix (Applied Biosystems) and 6·2 μL of sterile deionized water. The cycling conditions were set as follows: initial denaturation step at 95 °C for 10 min to activate the AmpliTaq® DNA Polymerase, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s. At the end of PCR amplification, a melting curve analysis was performed immediately to confirm the specificity of the reactions. Baseline and threshold cycles (Ct) were automatically determined using Real-Time PCR System software. PCR reactions were run in triplicate for each sample in three independent experiments. Relative expression was calculated using the comparative cycle threshold method (Livak and Schmittgen, 2001). The transcript abundance data were normalized by the average transcript abundance of two endogenous control genes, *ELFIA* and *ACTB*, between each sample.

Bioinformatic analysis

Primers were designed with the Primer3 software v.4.0.0 (http://primer3.wi.mit.edu/; Rozen and Skaletsky, 2000). The search for similarity among sequences was conducted by the BLAST service from the National Center for Biotechnology Information (NCBI, USA) (http://blast.ncbi.nlm.nih.gov/Blast.cgi; Altschul *et al.* 1990). The nucleotide to aminoacid sequence translation was made using the Translate Tool (http://web.expasy.org/translate/; Gasteiger *et al.* 2003). Multiple alignments of the nucleotide and amino acid sequences were performed using the multiple sequence alignment program ClustalW (http://www.genome.jp/tools/clustalw/; Larkin *et al.* 2007). The molecular weight and theoretical isoelectric point were predicted by ProtParam tool (http://web.expasy.org/protparam/; Gasteiger *et al.* 2005).

Statistical analysis

Expression data of *RmATG3, RmATG4* and *RmATG6* were analyzed by one-way ANOVA using Statgraphics™ 5.1 software. Tukey's test was used to determine significant differences of each gene at different developmental stages and adult tissues in *R. microplus*. The data represent mean ± S.D. from three independent experiments.

RESULTS

RmATG3

We amplified a PCR product of 1125 bp length. The flanked region is 1085 bp, has an ORF at position 106–1092 and codes for a 328-amino acid polypeptide with a predicted molecular mass of 36·29 kDa and a pI of 4·57 (online Fig. S1). The deduced polypeptide appears to have no signal peptide. The protein is similar in length to Atg3 from *H. longicornis* and *I. scapularis*. The identified protein was queried against the non-redundant protein database using the BLAST tool. BLAST analysis showed 86·23, 80·49, 65·53, 64·70, 60·67 and 60·06% identities between RmAtg3 and HlAtg3 of *H. longicornis*, putative Atg3 of *I. scapularis* and Atg3 of *Spodoptera litura, Bombyx mori, Aedes aegypti* and *Drosophila melanogaster*, respectively. All compared sequences contain three predicted domains, the autophagocytosis associated protein (Atg3), N-terminal domain (Autophagy_N), autophagocytosis associated protein, active-site domain (Autophagy_act_C) and the autophagocytosis associated protein C-terminal (Autophagy_Cterm) domain (Fig. 1). The region of active-site cysteine residue is conserved between RmAtg3 and other Atg3s within Autophagy_act_C domain (Fig. 1).

RmATG4

We amplified a PCR product of 1263 bp length. The flanked region is 1221 bp and it has an ORF at position 13–1176 that codes for a 387-amino acid polypeptide with a predicted molecular mass of 44·30 kDa and a pI of 5·22 (online Fig. S2). No signal peptide was found in the predicted polypeptide. The protein is similar in length to Atg4 s from other species. The deduced sequence was subjected to BLAST analysis. Among sequences that produced significant alignments, RmAtg4 showed an identity of 82·94, 71·20, 56·84, 49·08 and 48·32% with HlAtg4 of *H. longicornis*, putative Atg4 of *I. scapularis*, cysteine peptidase 2 family C54 protein of *Tityus serrulatus*, cysteine protease ATG4A of *Coptotermes formosanus* and cysteine protease ATG4B of *Zootermopsis nevadensis*, respectively. All compared sequences contain a Peptidase_C54 domain. Putative active sites, cysteine (C), aspartic acid (D) and histidine (H) at positions 76, 270 and 272 of RmAtg4, respectively, are widely conserved in all Atg4 homologues (Fig. 2).

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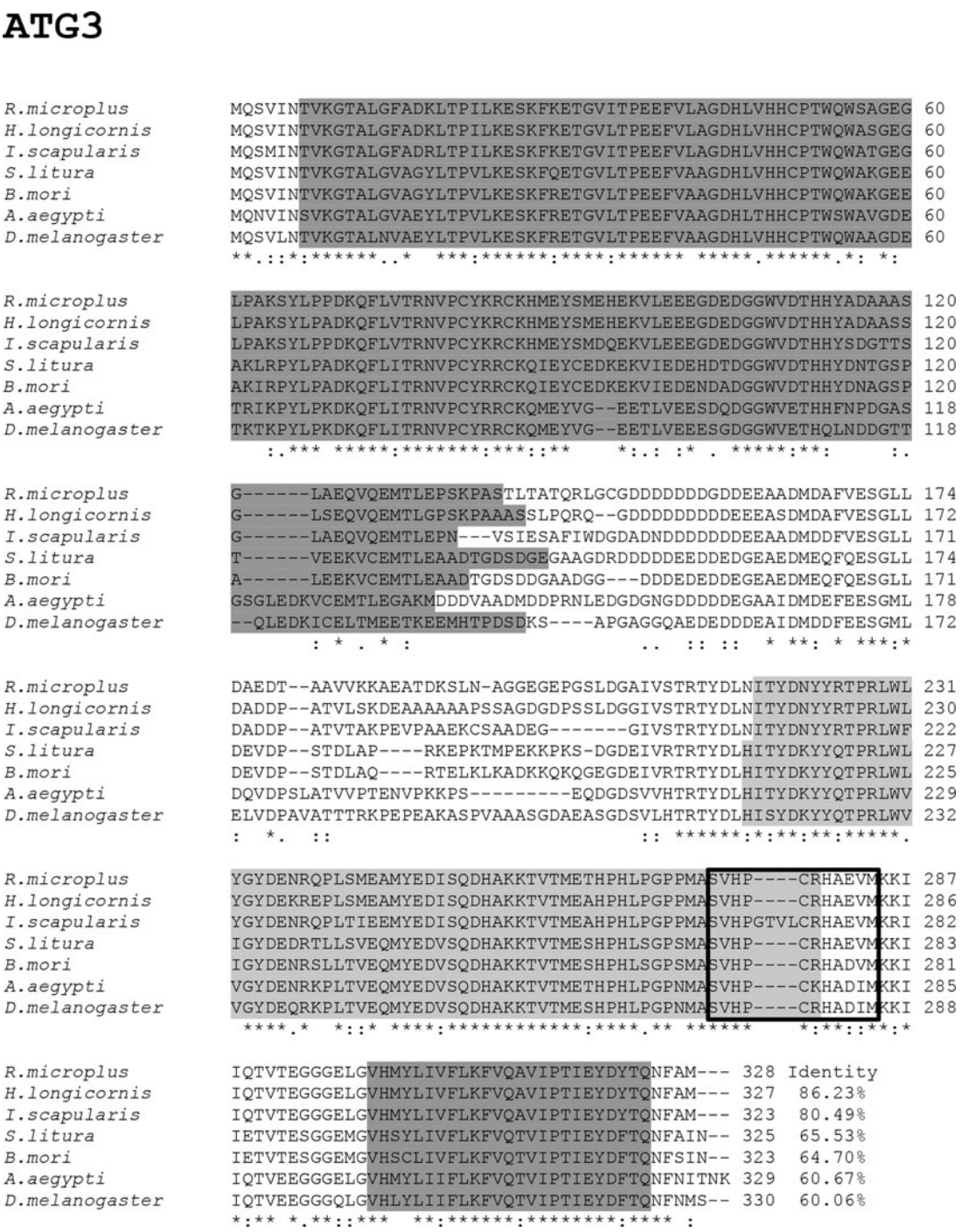

Fig. 1. Alignment of the deduced amino acid sequence of RmAtg3 (accession no. AKH60756), HlAtg3 of *Haemaphysalis longicornis* (accession no. BAI82575), putative Atg3 of *Ixodes scapularis* (accession no. XP_002402769), Atg3 of *Spodoptera litura* (accession no. AFS31123), Atg3 of *Bombyx mori* (accession no. NP_001135961), *Aedes aegypti* (accession no. XP_001657463) and *Drosophila melanogaster* (accession no. NP_649059). Autophagy_N, Autophagy_act_C and Autophagy_Cterm domains are shaded in gray dark, gray light and gray dark, respectively. The region of active-site cysteine residue 17 is boxed. An asterisk (*) indicates positions which have a single, fully conserved residue; a colon (:) indicates conservation between groups of strongly similar properties; a period (.) indicates conservation between groups of weakly similar properties.

RmATG6

We amplified a PCR product of 1575 bp length which was cloned and sequenced. The flanked region is 1537 bp, and it has an ORF at position 133–1470 that codes for a 445-amino acid polypeptide with a predicted molecular mass of 51·44 kDa and a pI of 5·03 (online Fig. S3). No signal peptide was found in the predicted polypeptide. The protein is 19 aa smaller than HlAtg4 from *H. longicornis* but 2 aa larger than that from *I. scapularis*. The deduced sequence of RmAtg6,

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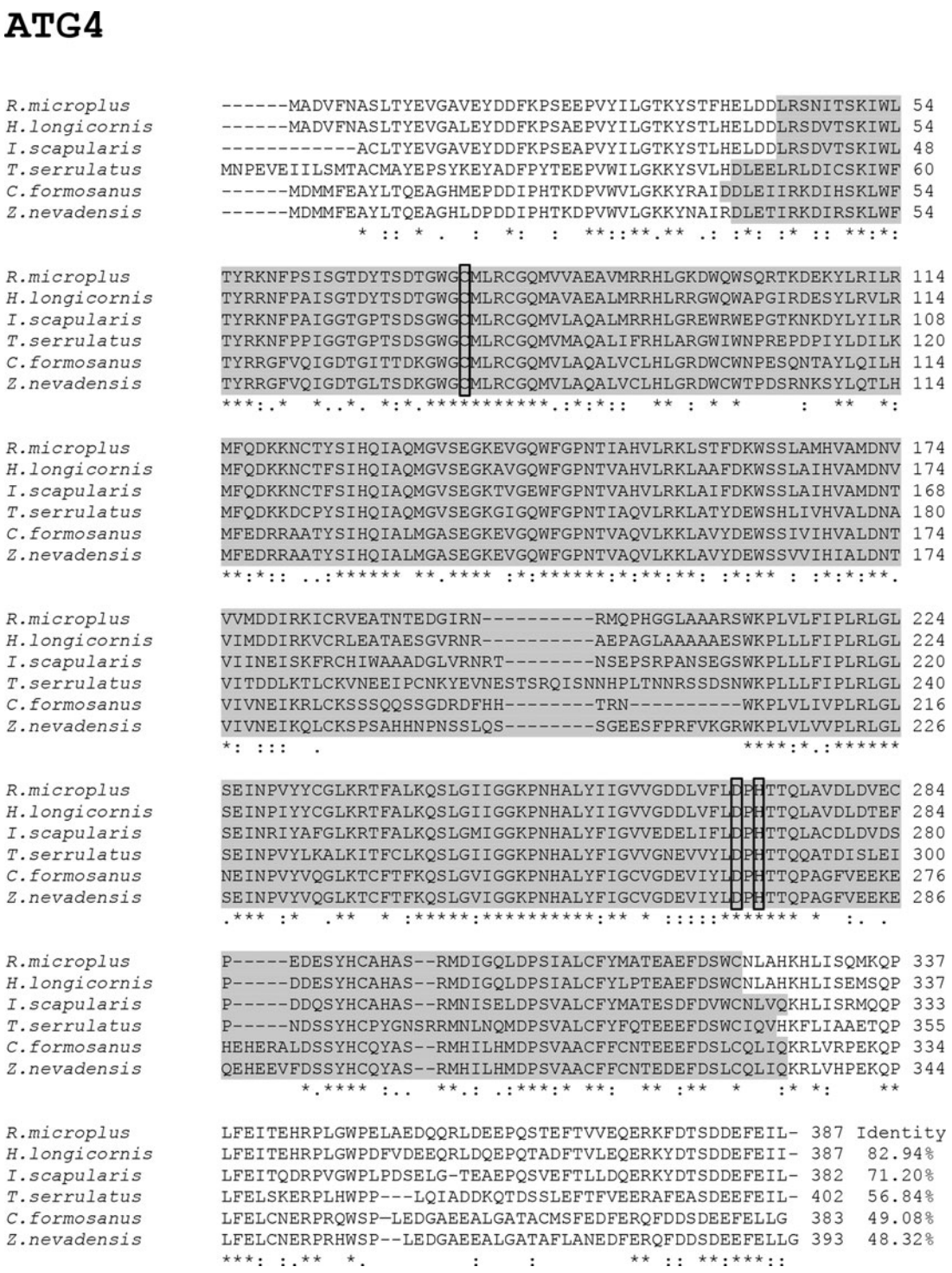

Fig. 2. Alignment of the deduced amino acid sequence of RmAtg4 (accession no. ALK28521), HlAtg4 of *Haemaphysalis longicornis* (accession no. BAI82576), putative Atg4 of *Ixodes scapularis* (accession no. XP_002434192), cysteine peptidase 2 family C54 protein of *Tityus serrulatus* (accession no. CDJ26740), cysteine protease ATG4A of *Coptotermes formosanus* (accession no. AGM32346) and Cysteine protease ATG4B of *Zootermopsis nevadensis* (accession no. KDR21327). Peptidase_C54 domain is shaded in gray. The putative active sites (Cys, Asp and His) are boxed. An asterisk (*) indicates positions which have a single, fully conserved residue; a colon (:) indicates conservation between groups of strongly similar properties; a period (.) indicates conservation between groups of weakly similar properties.

subjected to BLAST analysis, showed 84·94, 71·55, 51·68, 51·74 and 51·50% identities with HlAtg6 of *H. longicornis*, putative Beclin of *I. scapularis* and *Stegodyphus mimosarum*, and Beclin-1-like protein isoform X2 and X1 of *Camponotus floridanus* and *Cerapachys biroi*, respectively. All compared sequences contain the autophagy protein Apg6 domain (Fig. 3).

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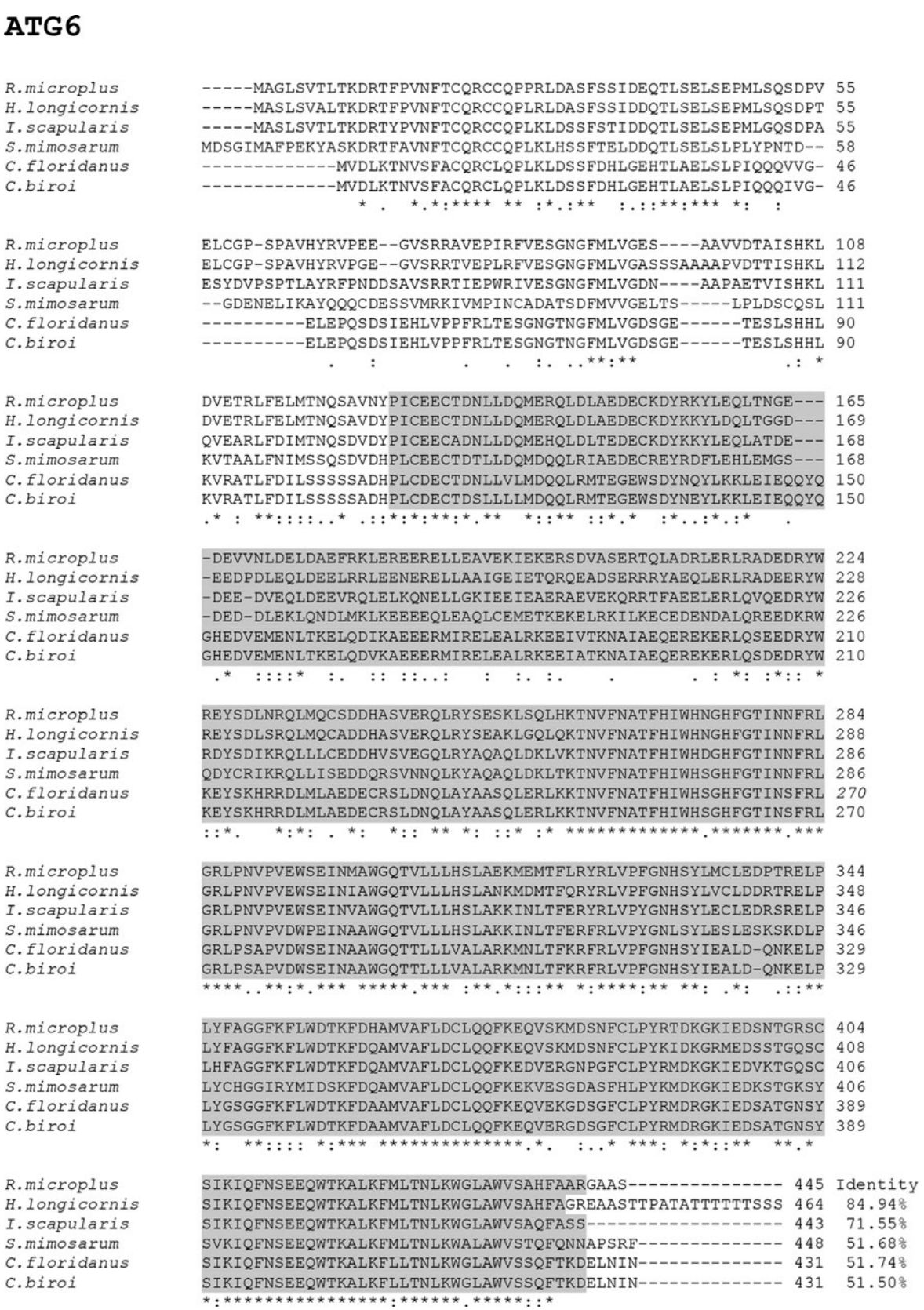

Fig. 3. Alignment of the deduced amino acid sequence of RmAtg6 (accession no. ALK28522), HlAtg6 of *Haemaphysalis longicornis* (accession no. BAK26532), putative Beclin of *Ixodes scapularis and Stegodyphus mimosarum* (accession no. XP_002414849 and KFM67317, respectively) and Beclin-1-like protein isoform X2 and X1 of *Camponotus floridanus* and *Cerapachys biroi* (accession no. XP_011257590 and XP_011344236, respectively). Autophagy protein Apg6 domain is shaded in gray. An asterisk (*) indicates positions which have a single, fully conserved residue; a colon (:) indicates conservation between groups of strongly similar properties; a period (.) indicates conservation between groups of weakly similar properties.

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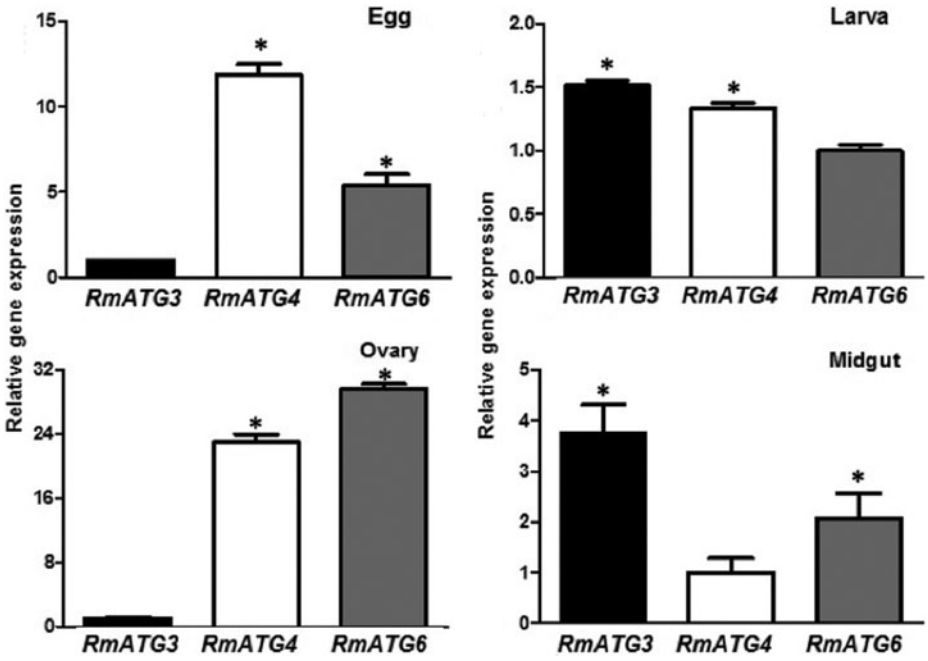

Fig. 4. *RmATG3, RmATG4* and *RmATG6* mRNA expression in different development stages of *Rhipicephalus microplus*. Real-time polymerase chain reaction (PCR) reactions were performed of total RNA from egg, larva and adult ovary and midgut tissues. Relative gene expression was calculated using the comparative cycle threshold method. The transcript abundance data were normalized by the average transcript abundance of two endogenous control genes, *Elongation factor 1-α* (*ELFIA*) and *β-actin* (*ACTB*) between each sample. Data represent the mean ± S.D. from three independent biological replicates. Be aware that each graph on the *y*-axis has a different scale.

Expression of RmATG genes in different development stages of R. microplus

The expression of *RmATG* genes showed a different pattern among development stages (Fig. 4). In midgut, *RmATG3* expression was 2-fold higher than in ovary, and egg and larva stages. In egg and ovary, *RmATG4* was overexpressed ~10-fold and 20-fold, respectively, compared to larva and midgut. Finally, *RmATG6* showed the highest expression level in ovary, followed by egg, midgut and larva.

DISCUSSION

In a previous work, we reported the molecular characterization of *RmATG8a* and *RmATG8b* genes and predicted the existence of the putative *ATG* genes *ATG3, ATG4* and *ATG6* in *R. microplus* (Flores *et al*. 2014). To give continuity on those findings, in this work we reported the complete sequences and expression analyses of *RmATG3, RmATG4* and *RmATG6*, providing new insights into autophagy in *R. microplus*. It is known that autophagy is activated in many species firstly by starvation conditions. The process involves the participation of multiple ATG proteins. To date, over 30 *ATG* genes, which are involved in various subtypes of macroautophagy, have been identified in yeast (Xie and Klionsky, 2007). Among *ATG* genes one subset, composed of 17 genes, is required for autophagosome formation. However, few *ATG* genes have been characterized in ticks. The genes *HlATG3, HlATG4, HlATG6, HlATG8* and *HlATG12* were recently characterized in *H. longicornis* ticks, while at least seven putative *ATG* genes (*ATG3, ATG5, ATG6, ATG7, ATG8, ATG13* and *ATG16*) have been found in *I. scapularis* ticks (Umemiya-Shirafuji *et al*. 2010).

ATG3, ATG4 and *ATG6* gene products, among others, are involved in autophagosome formation. Upon autophagosome induction ATG6 is recruited for vesicle nucleation (autophagosome biogenesis), while ATG3 and ATG4 participate in the elongation step (autophagosome maturation).

Even though the autophagy process is regulated by many ATG proteins, several studies have revealed that a complete set of ATGs may not be necessary in all organisms; some ATG proteins are not essential for starvation-induced autophagy, as is the case of the homologs ULK1 and ULK2 in the chicken, or perform a dual function; for example, in *B. mori* and other lepidoptera insects it is hypothesized that the function of ATG10 could be compensated for by ATG3 (Zhang *et al*. 2009; Mizushima and Sahani, 2014). Given the central role that ATG3, ATG4 and ATG6 play in the autophagosome formation, the characterization of their genes provides crucial information to better understand the autophagy process in *R. microplus*.

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The autophagy pathway is not only involved in starvation, but can also act in such diverse processes as cell death, immunity, embryogenesis, development, growth and nutrient utilization (McPhee and Baehrecke, 2009). In this study, the expression levels of *RmATG4* and *RmATG6* genes were found elevated in egg and ovary tissue, while *RmATG3* showed lower expression; these results are consistent with previous studies in *H. longicornis* tick, excepting the low expression of *RmATG3* (Kawano *et al*. 2011; Umemiya-Shirafuji *et al*. 2014). Our results, showing a similar gene expression pattern, suggest an involvement of the autophagy process in embryogenesis of *R. microplus*, as previously has been described in *H. longicornis*, where the silencing of *ATG6* mRNA affected the reproductive and ovipositional systems (Kawano *et al*. 2011).

To deepen the study of autophagy in *R. microplus*, a combination of methods such as real-time PCR, western blotting, immunostaining and transmission electronic microscopy, as well as RNAi-mediated gene silencing to analyze the function of *ATG* genes, are recommended. Moreover, a *R. microplus* genome sequencing project is an urgent need to characterize the complete set of genes that regulate autophagy in this tick.

SUPPLEMENTARY MATERIAL

The supplementary material for this article can be found at http://dx.doi.org/10.1017/S0031182016001542.

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