The impact of RNA interference of the subolesin and voraxin genes in male *Amblyomma hebraeum* (Acari: Ixodidae) on female engorgement and oviposition.

ALEXANDER SMITH[a], XIUYANG GUO[a,d], JOSÉ DE LA FUENTE[b,c], VICTORIA NARANJO[c], KATHERINE M. KOCAN[b], W. REUBEN KAUFMAN[a,*]

[a] Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada, T6G 2E9

[b] Department of Veterinary Pathobiology, Center for Veterinary Health Sciences, Oklahoma State University, Stillwater, OK 74078, USA

[c] Instituto de Investigación en Recursos Cinegéticos IREC (CSIC-UCLM-JCCM), Ronda de Toledo s/n, 13071 Ciudad Real, Spain

[d] Current address: Section of Rheumatology, Department of Internal Medicine, Yale University, New Haven, CT 06520-8031 USA

[*]Corresponding author. Z 606 Biological Sciences Building, University of Alberta, Edmonton, Alberta, Canada, T6G 2E9. Tel.: +1 780 492-1279, Fax: +1 780 492-9234.
Email address: reuben.kaufman@ualberta.ca

Running title: Effect on tick engorgement and fecundity by RNAi of two genes.

Abstract

Reducing or replacing the use of chemical pesticides for tick control is a desirable goal. The most promising approach would be to develop vaccines that protect hosts against tick infestation. Antigens suitable for the development of anti-tick vaccines will likely be those essential for vital physiological processes, and in particular those directly involved in feeding and reproduction. In this study genes from *Amblyomma hebraeum* Koch that encode for subolesin and voraxin were studied in male ticks by RNA interference (RNAi). Males (unfed or fed) were injected with dsRNA of (1) subolesin, (2) voraxin, (3) subolesin plus voraxin or (4) injection buffer, after which they were held off-host overnight and then allowed to feed on rabbits together with normal female *A. hebraeum.* Females that fed together with male ticks injected with subolesin or subolesin+voraxin dsRNA had a higher rate of mortality, weighed substantially less and produced a smaller egg mass than the controls. However, females feeding with males injected with voraxin dsRNA alone were not significantly different from the controls with respect to mortality, engorged weight or fecundity. However, as assessed by semi-quantitative RT-PCR, voraxin was not silenced in this study, the reasons for which remain unknown. The results of this study suggest that *A. hebraeum* subolesin is worthy of further testing as a candidate tick vaccine antigen.

Key words: ixodid tick, *Amblyomma hebraeum*, voraxin, subolesin, 4D8, midgut, salivary gland, testis, spermatozoa, RNA interference, anti-tick vaccine

INTRODUCTION

Ticks are major vectors of disease to humans and domestic animals (Parola and Raoult 2001; Peter et al. 2005). Notably, ticks and tick-borne pathogens have a negative impact on cattle production, with annual economic loss worldwide estimated at hundreds of millions of dollars due to direct effects of the tick infestations as well as the diseases caused by tick-borne pathogens (Peter et al. 2005). Along with the impact of tick feeding itself (reduced weight gains and milk production), cattle also suffer from diseases caused by the pathogens transmitted by *A. hebraeum* including *Ehrlichia* (formerly *Cowdria) ruminantium* (heartwater disease; Norval et al. 1989; Norval 1990). *A. hebraeum* is also the principal vector of *Rickettsia africae*, the agent of African tick bite fever (Kelly and Mason 1991; Norval et al. 1989).

Presently, acaricides constitute a major component of integrated tick control programs. However, use of acaricides is often accompanied by selection of acaricide-resistant ticks, environmental contamination, and contamination of milk and meat products with drug residues (Graf et al. 2004). Novel cost-effective strategies are therefore urgently needed for control of ticks that avoid the drawbacks of acaricides.

Use of vaccines has proven to be effective for controlling selected tick species on cattle (de la Fuente and Kocan 2003; Willadsen 2004). A tick vaccine, based on the midgut antigen, Bm86, has been used for over ten years in selected integrated programs for control of the cattle tick, *Rhipicephalus* (*Boophilus*) *microplus*, a species prevalent in Africa, Asia, Australia, the Caribbean and South America. Use of this vaccine has resulted in markedly reduced severity of tick infestations, a reduction in the incidence of some tick-borne diseases, and has reduced the numbers of required acaricide applications (reviewed by de al Fuente et al. 2007). Because these commercial vaccines, based on Bm86 (TickGard™ in Australia and GAVAC™ in the Caribbean and South America), are labeled for control of *R. (Boophilus) spp.* only (reviewed by

de la Fuente et al. 2007), there is an ongoing search for vaccine antigens useful for other tick species.

The complex feeding cycle of female *Amblyomma hebraeum* Koch requires up to 14 days of on-host feeding for repletion, and thus allows for considerable exposure of ticks to vaccine-derived antibodies during the blood meal. A feeding lesion is established during the preparatory phase (24 to 36 h), which is followed by a slow feeding phase (up to 10 d), during which the females undergo an approximate 10-fold weight gain. Mating occurs on the host and is required for full engorgement. The final, rapid feeding phase (up to 24 h) results in a further 10-fold increase in weight gain. Initiation of the rapid phase of feeding requires an 'engorgement factor' produced in the male gonad and transferred to the female during copulation (Pappas and Oliver 1972; Kaufman 2007). This engorgement factor, called 'voraxin', comprises two proteins, voraxinα and voraxinβ (Weiss and Kaufman 2004).

Immunization of a rabbit with recombinant voraxin caused a 74% reduction in the number of females that fed to engorgement and that ultimately oviposited (Weiss and Kaufman 2004). Feeding success of female ticks on the immunized rabbit fell into two categories: eight ticks achieved normal engorged weights and oviposited viable eggs. Twenty-three ticks, however, achieved only 4-5% of the normal engorged weight, and so were too small to oviposit.

The tick-protective antigen, subolesin (initially named '4D8'), was recently discovered in *Ixodes scapularis* by use of expression library immunization and sequence analysis of expressed sequence tags in a mouse model of tick infestations (Almazán et al. 2005a; Almazán et al. 2003a,b; Almazán et al. 2005b). Vaccine trials in mice, rabbits and sheep using recombinant subolesin resulted in significant reductions in larval, nymphal and adult *I. scapularis* infestations (Almazán et al. 2005a). While the subolesin gene is highly conserved among diverse ixodid tick species, the function of its expressed product is not known. Silencing of subolesin by RNA interference (RNAi) in *I. scapularis*, *A. americanum*, *Rhipicephalus*

sanguineus, *Dermacentor variabilis* and *D. marginatus* resulted in markedly reduced feeding success, subsequent oviposition, and survival after feeding (de la Fuente et al. 2006a). Male ticks in which the subolesin gene was silenced were unable to successfully mate with females, thus preventing the rapid stage of engorgement and oviposition (de la Fuente et al. 2006b).

Collectively, the foregoing suggest that subolesin and/or voraxin might show promise as candidate antigens for use in development of tick vaccines. In this study, we used RNAi to test the effect of silencing the expression of voraxin, subolesin or both together in male *A. hebraeum*.

MATERIALS AND METHODS

Ticks and hosts

Adult *A. hebraeum* used for these studies were from a laboratory colony at the Department of Biological Sciences, University of Alberta. When off hosts, the ticks were held in the dark, at 26°C, and high relative humidity. For feeding, ticks were confined to cloth-covered foam arenas attached with latex adhesive (Roberts 8502 Latex, Bramalea, Ontario, Canada) to the shaven backs of rabbits as described by Kaufman and Phillips (1973). The arenas were divided into two compartments on selected rabbits to allow for simultaneous feeding of two distinct experimental groups. The use of rabbits for this research project was reviewed and approved by the Biosciences Animal Policy and Welfare Committee, University of Alberta, which functions according to the current guidelines established by the Canadian Council on Animal Care.

Cloning of A. hebraeum subolesin and dsRNA synthesis for subolesin and voraxin

The *A. hebraeum* subolesin cDNA was amplified by RT-PCR, according to procedures reported previously, using oligonucleotide primers 4D8R5 and 4D833 (Table 1, de la Fuente et al. 2006a). Oligonucleotide primers containing T7 promoter sequences for *in vitro* transcription and synthesis of dsRNA were synthesized for amplification of the genes encoding *A. hebraeum*

subolesin, voraxinα and voraxinβ (Table 1). RT-PCR and dsRNA synthesis reactions were performed according to procedures described previously for other tick species (de la Fuente et al. 2005) using the Access RT-PCR system (Promega, Madison, WI, USA) and the Megascript RNAi kit (Ambion, Austin, TX, USA). The dsRNA was purified and quantified by spectrophotometry (260 nm) using a Nanodrop ND-1000 spectrophotometer. The Genbank accession number for *A. hebraeum* subolesin is EU262598. Genbank accession numbers for *A. hebraeum* voraxinα and voraxinβ are AY442319 and AY442320, respectively.

RNAi in unfed male ticks

In the first round of tick feeding, four treatment groups of 40-48 males ticks each were used: (1) subolesin dsRNA, (2) voraxinα & voraxinβ (hereafter referred to simply as 'voraxin') dsRNA, (3) subolesin + voraxin dsRNA and (4) control ticks injected with injection buffer (10 mM Tris-HCl, pH 7, 1 mM EDTA) alone. The ticks from each treatment group were fed on two rabbits (half of the ticks on each), and each rabbit served as a host for two treatment groups, each group feeding in separate halves of the chamber.

For RNAi, unfed male *A. hebraeum* were injected with approximately 1 µl of dsRNA (2.4-3.2 x 10^{11} molecules/µl), an amount that has proved successful in our earlier RNAi studies. Injection of ticks was done in the lower right quadrant of the ventral surface. The exoskeleton was first pierced with the tip of a 30g needle to create an opening, and then the dsRNA was injected through this opening into the hemocoel using a Hamilton® syringe fitted with a 33g needle. After injection, the males were held overnight in the colony incubator before being placed on rabbits in the appropriate feeding chamber. One day after the males had attached to the rabbits, an equal number of unfed, virgin females was added to each feeding chamber. Feeding progress in the females was monitored for up to 20 days, after which all remaining females were removed, weighed and stored in individual gauze-covered plastic vials in the colony incubator for oviposition. In this study we define ‘engorgement’ as females that detached

from the host spontaneously within the 20-day feeding period; ticks that were forcibly removed from the host on the 20th day are defined as 'partially fed'. At 42-51 days after engorgement or removing ticks from the rabbits, the egg mass weight produced by each female was recorded, because by this time, under these holding conditions, oviposition in *A. hebraeum* is known to have stopped (Friesen and Kaufman 2002). Hatching success for individual egg masses was recorded 36 days after oviposition. Five to ten male ticks from each group were used for histological studies, and an additional five ticks from each group were used to determine gene silencing by semi-quantitative RT-PCR (see below).

Second feeding of previously fed males

Some of the surviving dsRNA-injected male ticks (nine for the subolesin group and 15 for each of the other groups) were placed on a second group of tick-naïve rabbits and allowed to feed again for 23 days with an equal number of unfed females, in order to see whether gene silencing in the males was prolonged beyond a single feeding cycle. Engorgement success, oviposition, hatching and gene silencing in the males were evaluated in the same manner as during the first round of tick feeding.

Second feeding of females

To assess the duration of the inhibitory effect of dsRNA-injected males on the females, a group of female ticks that had been previously fed with males injected with subolesin or subolesin+voraxin dsRNAs, and did not enter the rapid phase of feeding, were placed on a fresh, tick naive rabbit and allowed to feed with normal males. Feeding success and fecundity of these females were monitored as described above.

RNAi in fed male ticks

For reasons outlined in Results (*Ticks pre-fed before injection of dsRNA*) we also attempted RNAi by injecting dsRNA into fed males as follows: Three groups of 15 male ticks were allowed to feed for one, two, or three days on tick-naïve rabbits, then injected with 3 µl (2.0 x 10^{12} molecules/µl) of voraxin dsRNA or injection buffer alone as described above, held

overnight in the colony incubator, and then returned to the rabbits on which they had fed previously. One day after each group of males had reattached, an equal number of females was added to each feeding chamber on the rabbit. Feeding progress was monitored for up to 17 days, after which all remaining females were removed and weighed. Females were stored in individual gauze-covered plastic vials in the colony incubator, and total egg mass of each female was weighed 48-56 days post removal from the host. Subsequent hatching success was recorded for each female 66-71 days later. Five males from each group were used to determine voraxin silencing by semi-quantitative RT-PCR (see below). Ten unfed male ticks were injected with 3 µl (1.2 x 10^{12} molecules/µl) of subolesin-, voraxin-, or both- dsRNAs, or injection buffer, according to the protocol above. These injected males were allowed to feed on a tick-naïve rabbit, half of each group were allowed to feed for 5 days and the other half were allowed to feed for 10 days, before being forcibly removed and assayed for gene expression levels by semi-quantitative RT-PCR (see below).

Determination of subolesin and voraxin mRNA levels after RNAi

Subolesin and voraxin mRNA levels after RNAi in male ticks was measured by semi-quantitative RT-PCR as follows: Total RNA was extracted from testes and midguts of male ticks after 5, 10, and 18 days of feeding and reverse-transcribed according to the protocols outlined previously. Subolesin, voraxinα, voraxinβ and 16S rRNA levels were analyzed using using gene-specific primers (Table 1) in a 50 µl reaction mixture (1 µl cDNA, 1.5 mM $MgCl_2$, 25 mM KCl, 5 nM EDTA, 10mM Tris-HCl, pH 9.2, 50 nM each deoxynucleoside triphosphate [dNTP]), 0.5 U Hot-Start Taq DNA polymerase (Fermentas, Burlington, ON, Canada, 0.5 µM each primer). All reactions were incubated at 94°C for 5 min, then 35 cycles of (94°C, 30 s; 60°C, 60 s; 72°C, 2 min for denaturation, annealing and extension conditions, respectively), followed by 72°C, 10 min. Control reactions were performed using the same procedures but without reverse transcriptase to test for DNA contamination in the RNA preparations and without DNA to detect contamination of the PCR. PCR products were electrophoresed on 1% agarose gels to check

the size of amplified fragments by comparison to a DNA molecular weight marker (GeneRuler 1kb DNA Ladder Plus, Fermentas).

Light microscopy

Selected male ticks collected after the first and second rounds of tick feeding (2-5 ticks per treatment group) were fixed and processed for light microscopy studies of resin-embedded sections. Tick fixation and processing were done according to the procedures of Kocan et al. (1980). Briefly, ticks were cut in half at the midline using a razor blade, separating the right and left halves. The two tick halves were fixed immediately in individual vials containing 2% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.4) and held at 4^0C until further processing. The tick halves were post-fixed in 2% cacodylate-buffered osmium tetroxide (pH 7.4), dehydrated in a graded series of ethanol (70% – 100%), and embedded in epoxy resin. Sections (1 µm) were cut with an ultramicrotome and stained with Mallory's stain (Richardson et al. 1960). Photomicrographs were recorded with a light microscope equipped with a 3-chip digital camera.

Statistical analysis

The weight of females after feeding and the weight of egg masses were compared using the Kruskal-Wallis test, the nonparametric analogue of the one-way ANOVA, followed by pairwise comparisons. Tick mortality and engorgement rates were recorded as the percentage of dead and engorged ticks, respectively, to the total number of ticks fed after 20 days (first round of feeding) or 23 days (second round of feeding). Tick mortality, engorgement and hatching success rates were compared by using Fisher's Exact test. All statistical analyses were performed using Stata statistical software (College Station, TX: StataCorp LP).

RESULTS

The first round of tick feeding

The mortality rate of males injected with subolesin dsRNA (53%) was significantly higher compared to all other treatments ($p < 0.001$), whereas significant differences in tick mortality rates were not observed between the voraxin (0%), subolesin+voraxin (7%) and control groups (2%; $p = 0.105$ and $p = 0.355$, respectively; Fisher's exact test; Table 2).

Representative samples of the ticks in the four treatment groups that engorged or were removed after 20 days of feeding are shown in Fig. 1. In this figure it is visually apparent that females co-fed with subolesin dsRNA-treated or subolesin+voraxin dsRNA-treated males fed substantially less successfully than females co-fed with either the voraxin dsRNA-treated or the control males, and this impression was verified statistically in Table 2. All but one of the females that were fed with voraxin dsRNA-injected males or with control males fed to engorgement (98% and 100%, respectively; Table 2). On the other hand, very few of the females that fed with subolesin dsRNA-injected or subolesin+voraxin dsRNA-injected males engorged fully (10% and 0%, respectively; $p <0.001$; Table 2). Females co-feeding with either the subolesin or the subolesin+voraxin group had significantly lower body weights after feeding (309 mg and 187 mg, respectively) than either the voraxin or the control group (1813 mg and 1945 mg, respectively; $p <0.001$). There was no significant difference, however, between the mean weights of females feeding with subolesin dsRNA-treated males or with subolesin+voraxin dsRNA-treated males (309 mg and 187 mg, respectively ($p = 0.340$; Table 2).

The average weight of the egg masses produced by females from the subolesin and subolesin+voraxin groups (33% and 16% of female body weight (bw), respectively) was significantly lower than that of the control or voraxin group (54% and 48% bw, respectively; $p <0.001$). The average weight of the egg masses produced by females from the voraxin group (48% bw) was marginally lower than that of the control group (54%; $p = 0.042$). Viability of the eggs (i.e., hatching success) produced by females that fed with subolesin or subolesin+voraxin dsRNA injected males (50% and 0%, respectively) was significantly lower than that of eggs produced by females fed with buffer-injected or voraxin dsRNA-injected ticks (100% and 98%,

respectively; p <0.001). The proportion of eggs that hatched from egg masses produced by females in the subolesin+voraxin group (0%) was significantly lower than that of the subolesin group (50%; p = 0.033; Table 2).

The second round of tick feeding

Males available after the first round of feeding were removed, held overnight in the colony incubator and then allowed to feed on rabbits for 24 days with a fresh batch of unfed virgin females, in order to determine the duration of RNAi in the males. Males that had been injected previously with subolesin or subolesin+voraxin dsRNAs, and allowed to feed a second time with unfed untreated females, had significantly higher mortality rates than did the buffer-injected controls (78%, 53% and 7%, respectively; p = 0.001 and p = 0.014, respectively; Table 2). Males from the subolesin+voraxin group also had a significantly higher mortality rate during the second round of feeding compared to the first (53% vs 7%, p < 0.001). Only 29% of females fed with males injected previously with subolesin dsRNA, and none of females fed with subolesin+voraxin group ticks, fed to repletion. Both of these groups fed significantly less successfully than either the voraxin dsRNA injected (p = 0.017 and p < 0.001, respectively) or control groups (86% and 80% respectively; p = 0.052 and p <0.001 for subolesin and subolesin+voraxin, respectively; Table 2).

Females from the subolesin and the subolesin+voraxin groups (701 mg and 369 mg, respectively) weighed significantly less than those from either the voraxin (1967 mg, p = 0.007 and p <0.001, respectively) or control group (1844 mg, p = 0.020 and p <0.001, respectively). Weights of the females from the subolesin and the subolesin+voraxin groups (701 mg and 369 mg, respectively) were not significantly different from each other (p = 0.193; Table 2).

The mean egg mass weights from both the subolesin and subolesin+voraxin groups (17% bw and 9% bw, respectively) were significantly lower than that of the control (34% bw, p = 0.045 and p = 0.004, respectively). The difference between the subolesin and subolesin+voraxin groups was not statistically significant (17% and 9%, respectively; p = 0.248; Table 2).

Hatching success in the subolesin and subolesin+voraxin groups (20% and 0%, respectively) was significantly lower than that of the voraxin (100%, $p = 0.002$ and $p < 0.001$, respectively) or control groups (92%, $p = 0.010$ and $p < 0.001$, respectively; Table 2), but were not significantly different from each other ($p = 0.455$). In brief, the effect of dsRNA treatment of males on female feeding success and fecundity was maintained over at least two feeding cycles by the males.

Determination of subolesin and voraxin mRNA levels after RNAi

Semi-quantitative RT-PCR analysis of gene expression levels showed a reduction of the subolesin transcript compared to the voraxin dsRNA-injected or buffer- injected control males after 5 or 10 days of feeding (Fig. 2). Levels of both voraxinα and voraxinβ in the voraxin dsRNA injected males were not reduced relative to the control ticks. Levels of voraxinβ were low for all samples and could not be amplified from the subolesin-silenced males, but could be amplified from the males injected with subolesin+voraxin dsRNAs (Fig. 2).

Second round of feeding of the females that had not engorged after the first round of feeding

We next tested whether females, failing to engorge when fed with gene-silenced males (subolesin or subolesin+voraxin) during the first round of feeding, were subsequently able to engorge if co-fed with normal males on a tick-naïve host. The data are found in Table 2, "2nd feeding of females". Mortality of these females during the course of feeding was 0%. Engorgement occurred in 80% and 89% of these females, vs 10% and 0% during their first opportunity to engorge. Similarly, these females achieved engorged weights within the normal range (1353 mg and 1652 mg, respectively). Their egg masses (35% bw and 38% bw, respectively) were within the normal range, and hatching success (100% and 96%, respectively) was much higher than that of similarly treated females during the first round of feeding (50% and 0%, respectively; Table 2).

Ticks pre-fed before injection of dsRNA

Injection of voraxin dsRNA into unfed males failed to inhibit engorgement in co-feeding females (Table 2). One possibility for this negative result relates to the fact that voraxin is up-regulated during feeding (Weiss and Kaufman 2004), and so voraxin mRNA was unlikely to have been present in unfed males for the dsRNA to act on. We thus repeated these experiments with fed male ticks. We injected voraxin dsRNA in males fed for 1, 2 or 3 days, and then allowing them to feed with females. Most of these females engorged and produced normal egg masses (Table 2). Semi-quantitative RT-PCR analysis of gene expression levels did not show substantial decreases in levels of the voraxin transcripts in the males injected with voraxin dsRNA (Fig. 2).

Light microscopic changes in tick salivary gland, midgut and testis after RNAi

Noticeable light microscopic differences were observed in salivary gland, midgut, and testis following RNAi of subolesin or subolesin+voraxin, as compared to the injection-buffer controls, and these changes were similar after the first and second tick rounds of feeding (Fig. 3). Injection of ticks with voraxin dsRNA alone did not appear to affect these tissues, which were histologically similar to those of the controls. However, male ticks injected with subolesin or subolesin+voraxin dsRNA had salivary gland acini that were crenated and appeared smaller in diameter Fig. 3b) compared to the injection-buffer controls (Fig. 3a). Tick midgut appeared to be profoundly affected in both the subolesin and subolesin+voraxin groups, and showed signs of advanced degeneration (Fig. 3d) compared to the control (Fig. 3c). Few gut epithelial cells were attached to the basement membrane, and sloughed cells and cellular debris were seen within the gut lumen (Fig. 3d). In the testis of males injected with subolesin or subolesin+voraxin dsRNA, few prospermia (mature spermatids) were seen (Fig. 3f), and these prospermia appeared deformed relative to those from controls (Fig. 3e). Cellular debris and clear spaces surrounded the scattered prospermia (Fig. 3f).

DISCUSSION

The focus of this study was attempting to silence targeted genes in male ticks, and then assessing the ability of these males to mate successfully with normal females, leading the latter to engorge and oviposit. In most ixodid tick species, copulation occurs only on the host during feeding; copulation is necessary for females to achieve full engorgement and subsequently oviposit (reviewed by Kaufman 2007). Transfer of voraxin via the spermatophore is required for female feeding and oviposition in *A. hebraeum* (Weiss and Kaufman 2004). The importance of voraxin as an engorgement stimulus in *A. hebraeum* was demonstrated by marked inhibition of engorgement in females feeding on a rabbit that had been immunized against the two voraxin proteins (Weiss and Kaufman 2004).

Whereas homologues of voraxin are suspected in other ixodid tick species, they have not yet been widely reported, the exceptions being three entries in GenBank: a peptide from *D. variabilis* showing 83% identity to voraxinα of *A. hebraeum* and two EST entries from the BmGI dataset for *Rhipicephalus (Boophilus) microplus,* showing 77% and 76% similarity to voraxinα of *A. hebraeum.* To date, no homologues of voraxinβ have been reported. However, among tick species in which partially fed virgin ticks do not engorge fully, there is substantial inter-specific variability in the extent to which they feed. For example, the approximate maximum virgin-to-mated weight ratio has been reported for laboratory-reared ticks as follows: *A. americanum* (~5%), *A. hebraeum* (~10% for the vast majority of virgins and ~20% for a small minority), *R. sanguineus* (~17%), *D. andersoni* (~34%), *D. variabilis* (~35%), and *Hyalomma anatolicum* (up to 39%) (reviewed by Kaufman 2007). In *A. hebraeum*, physiological and behavioral changes were observed to occur when females exceed ~10X the unfed weight, a transition that has been called the critical weight (CW) (Harris and Kaufman 1984). Below the CW, (1) females will reattach to a host if given the opportunity, (2) the salivary glands will not undergo autolysis within 4 days (mated) or 8 days (virgin), and (3) vitellogenesis is curtailed and oviposition does

not occur. Females above the CW (1) will not reattach to a host if given the opportunity, (2) the salivary glands undergo degeneration (4 days for mated and 8 days for virgins) and (3) vitellogenesis and oviposition occurs (Kaufman and Lomas 1996; Lomas and Kaufman 1999). The exact value of the CW depends on which parameter is used to measure it. Thus, the CW as measured by reluctance to reattach to the host was 9X the unfed weight, while for hemolymph ecdysteroid titer, salivary gland degeneration, ovary weight, oocyte length and oocyte vitellin content the CW was 10X, 10X, 12X, 12X and 13X, respectively (Weiss and Kaufman 2001). The biological significance of these small but distinct differences is not known. Although in *A. hebraeum*, virgin females rarely feed above the minimum weight for laying eggs, this is not the case for at least some other species. Virgin *D. variabilis* and *D. andersoni*, which normally feed to ~35 X the unfed weight, do lay (infertile) eggs at that size (personal communications from: Dr. Dan Sonenshine, Old Dominion University, Norfolk Virginia, USA for *D. variabilis* and Dr. Tim Lysyk, Agriculture and Agri-Food Canada, Lethbridge Alberta, for *D. andersoni*).

The effects of silencing the subolesin gene has been tested in *I. scapularis, A. americanum*, *R. sanguineus*, *D. variabilis*, *D. marginatus* and *R. microplus* (Almazán et al. 2003 a,b; 2005 a,b; de la Fuente et al. 2006 a,b; Nijhof et al. 2007; Kocan et al. 2007). The silencing of subolesin by RNAi resulted in production of sterile males that apparently did not mate successfully with females because these females failed to engorge, complete the rapid stage of engorgement or oviposit (de la Fuente et al. 2006b). Immunization of sheep with recombinant *I. scapularis* subolesin reduced larval, nymphal and adult tick infestations, further demonstrating the usefulness of this protein as a candidate vaccine antigen (Almazán *et al*. 2005a, b).

In this study, most females that fed together with males, in which subolesin or subolesin+voraxin were silenced by RNAi, failed to engorge (Table 2). The females that failed to engorge had unlimited opportunity to pair and copulate, but neither the frequency of pairing nor confirmation of spermatophore transfer from the male was confirmed. Therefore, the extent to which failure of the females to engorge was due a failure to copulate was not determined.

However, a previous study in *D. variabilis* demonstrated that subolesin dsRNA-treated males paired with females at a frequency similar to controls, but spermatophore transfer apparently still did not occur because the females did not engorge and produce egg masses (de la Fuente et al. 2006b). Injection of males with subolesin dsRNA in this study caused noticeable histological degeneration of the testis (Fig. 3). So even if pairing had occurred, inhibition of sperm production and spermatophore transfer were most likely the main reasons for the deleterious effects we observed here.

Males injected with subolesin dsRNA alone suffered significantly higher mortality compared to that suffered by males injected with subolesin+voraxin dsRNA (Table 2); we do not know the reason for this difference. One possible explanation is that the simultaneous injection of voraxin dsRNA offered some protection against the deleterious effects of subolesin dsRNA; if so, there is no obvious explanation for the mechanism of the hypothetical protection. The following data are not consistent with this hypothesis, however. Whereas males injected with subolesin or the subolesin+voraxin dsRNA suffered significantly different mortality, the females mated to the males of each group exhibited similar failures to feed to engorgement, lay normal egg masses, and produce viable larvae (Table 2). Clearly, more work is required to investigate this matter.

During the second round of male feeding on a tick-naïve rabbit, most females were still unable to feed successfully and oviposit (Table 2). One possibility for this failure could have been the high mortality of the males. Thus there were only two of the nine injected males alive and available for the seven females in the subolesin group, and seven males alive and available for the 14 females in the subolesin+voraxin group. However, the fact that both groups of females fared poorly to a similar degree, even though there were significantly more males available to the latter group of females, leads us to interpret these results as continued efficacy of gene silencing through a second round of feeding.

We expected that silencing of voraxin alone in male *A. hebraeum* would result in an inhibition of engorgement and subsequent oviposition in co-feeding females. However, the mean engorged weight of the females and the mean weight of the egg masses they laid were similar to those of the controls (Table 2). In other words, the voraxin genes were still being expressed, notwithstanding treatment with dsRNA on day 1, 2 or 3 of feeding (Table 2). Our semi-quantitative RT-PCR results confirmed that the voraxin genes were not silenced (Fig. 2), the reason(s) for which are not known. In contrast, expression of subolesin was reduced as measured by semi-quantitative RT-PCR (Fig. 2), and subolesin dsRNA-treatment of *A. hebraeum* led to the types of pathological effect shown to be associated with gene silencing in other tick species (reviewed by de la Fuente et al. 2007).

This study demonstrated the utility of RNAi to study the role of tick genes in tick biology. Although RNAi is not a direct predictor of the success of using gene products in a vaccine formulation, this technique does allow for study of the direct impacts of gene silencing.

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FIGURE LEGENDS

Fig. 1 Appearance of a representative sample of engorged ticks and of those removed after 20 days of feeding for the four treatment groups: (a) subolesin dsRNA, (b) voraxin dsRNA, (c) subolesin plus voraxin dsRNA, (d) injection-buffer control. Bars in all panels, 1 cm.

Fig. 2 Semi-quantitative RT-PCR to determine gene expression levels of subolesin, voraxin and 16S rRNA transcripts. The left column (numbers in parenthesis indicate size of gene product amplified by PCR reaction) shows levels of *subolesin*, *voraxin*α *voraxin*β or 16S rRNA present in male ticks injected with (top row) subolesin, voraxin, subolesin+voraxin dsRNAs or buffer and allowed to feed for 5 or 10 days. No differences in gene expression levels were observed between males fed for 5 or 10 days after injection (data not shown). The right column shows levels of *voraxin*α, *voraxin*β and 16S rRNA present in male ticks that were injected with voraxin dsRNA or buffer after 1, 2 or 3 days of feeding, then allowed to feed a further 18 days before being sacrificed for gene level analysis.

Fig. 3 Light photomicrographs of 1 µm plastic sections of salivary gland, midgut, and testis from male *A. hebraeum*. Photomicrographs are from representative ticks injected with subolesin dsRNA (n = 6), subolesin+voraxin dsRNAs (n = 7) or injection buffer alone (n = 10). In contrast to salivary glands from the control (injection buffer) ticks (Fig. a), the salivary gland acini from subolesin dsRNA treated ticks (Fig. b) were noticeably smaller, and many acini were crenated as evidenced by the smaller size and scalloped edges (small arrows). Differences in granule structure were not apparent between the granular cells of control and treated ticks. In the control midgut (Fig. c) large columnar digestive cells (CDC) were situated along the midgut basement membrane (BM), and contained numerous dark hematin granules. Midguts from subolesin or subolesin+voraxin silenced ticks (Fig. d) were largely filled with sloughed and degenerating cells (DC), many of which were not attached to the basement membrane (BM). These cells filled the midgut lumen (L) along with debris.

The control testis (Fig. e) was packed with large, mature prospermia. In contrast (Fig. f) testis from subolesin and subolesin+voraxin silenced males contained only scattered prospermia (small arrows) which appeared to be deformed. Cellular debris (CD) was seen surrounding the prospermia. All bars, 20 μm.

Table 1 List of oligonucleotide primers used in this study

Primer	Sequence (5'-3')	Purpose	Amplicon Size (bp)
4D8R5	GCTTGCGCAACATTAAAGCGAAC	subolesin cloning primer	466
4D833	TTTGGTCGTACGTAAACTTGACAAATGTG	subolesin cloning primer	
D8AAT75	TAATACGACTCACTATAGGGTACTGACTGGGATCCCCTGCACAGT	subolesin dsRNA synthesis	466
D8AAT73	TAATACGACTCACTATAGGGTACTCAAGCTTGGTGGAGAGCACG	subolesin dsRNA synthesis	
EFa5'	TAATACGACTCACTATAGGGTACTATGTTGATCACCAAGGACCTG	voraxinα dsRNA synthesis	384
EFa3'	TAATACGACTCACTATAGGGTACTCTATCGACCAGTGTCAAGCTC	voraxinα dsRNA synthesis	
EFb5'	TAATACGACTCACTATAGGGTACTATGGCGAAACAGGGACTTCTG	voraxinβ dsRNA synthesis	264
EFb3'	TAATACGACTCACTATAGGGTACTTCACCGCAGGCTCCCCAGG	voraxinβ dsRNA synthesis	
Sub93F	GACTGGGATCCCCTGCACAGTC	measure subolesin transcript levels	419
Sub94R	CAAGCTTGGTGGAGAGCACG	measure subolesin transcript levels	
VorA43R	CCACGCCTTGAGGAAAAGG	measure voraxinα transcript levels	311
VorA44F	GGCCGAGAACCTCGTCAA	measure voraxinα transcript levels	
VorB51R	TGCGGCGCTGGGAGTA	measure voraxinβ transcript levels	323
VorB87F	ATGGCGAAACAGGGACTTCTGAAG	measure voraxinβ transcript levels	
16S89F	CTGCTCAATGATTTTTTAAATTGCTGTGG	measure 16S rRNA transcript levels	456
16S90R	CCGGTCTGAACTCAGATCAAGT	measure 16S rRNA transcript levels	

Table 2 Effect of subolesin and voraxin silencing on *A. hebraeum* survival, feeding, and fecundity

dsRNA-treatment of males (and initial n)	Number of males dead/total (and %) after feeding	Number of females fed to repletion/total (and %)	Average weight of females after feeding (mg/tick) ± SEM (n)	Average weight (mg) of egg mass ± SEM (n)	Egg mass (% of female body weight) ± SEM (n)	Number of ticks producing viable larvae/total fed (and %)
1st round of feeding						
Subolesin (40)	21/40 (**53%**)**	4/39 (**10%**)**	**309** ± 82 (39)**	**401** ± 142 (10)**	**33** ± 5 (10)**	5/10 (**50%**)**
Voraxin (48)	0/48 (**0%**)	47/48 (**98%**)	**1813** ± 100 (48)	**885** ± 66 (48)*	**48** ± 2 (48)*	47/48 (**98%**)
Subolesin+Voraxin (44)	3/44 (**7%**)	0/44 (**0%**)**	**187** ± 24 (44)**	**71** ± 10 (11)**	**16** ± 2 (11)**	0/10 (**0%**)**
Control (46)	1/46 (**2%**)	46/46 (**100%**)	**1945** ± 81 (46)	**1069** ± 55 (46)	**54** ± 1 (46)	46/46 (**100%**)
2nd round of feeding						
Subolesin (9)	7/9 (**78%**)**	2/7 (**29%**)	**701** ± 257 (7)*	**248** ± 194 (6)*	**17** ± 8 (6)*	1/5 (**20%**)*
Voraxin (15)	1/15 (**7%**)	12/14 (**86%**)	**1967** ± 270 (14)	**950** ± 174 (14)	**40** ± 5 (14)	13/13 (**100%**)
Subolesin+Voraxin (15)	8/15 (**53%**)*	0/14 (**0%**)**	**369** ± 69 (14)**	**60** ± 16 (7)**	**9** ± 2 (7)**	0/6 (**0%**)**
Control (15)	1/15 (**7%**)	12/15 (**80%**)	**1844** ± 273 (15)	**746** ± 135 (13)	**34** ± 6 (13)	11/12 (**92%**)
2nd feeding of females[a]						
Subolesin (25)	0/25 (**0%**)	20/25 (**80%**)	**1353** ± 176 (25)	**633** ± 107 (22)	**35** ± 4 (22)	21/21 (**100%**)
Subolesin+voraxin (28)	0/28 (**0%**)	25/28 (**89%**)	**1652** ± 98 (28)	**679** ± 77 (28)	**38** ± 3 (28)	27/28 (**96%**)
Pre-fed before injection						
Voraxin - 1 day (15)	1/15 (**7%**)	15/15 (**100%**)	**1541** ± 88 (15)	**759** ± 56 (15)	**49** ± 1 (15)	15/15 **(100%)**
Voraxin - 2 days (15)	0/15 (**0%**)	15/15 (**100%**)	**1575** ± 180 (15)	**837** ± 117 (15)	**50** ± 3 (15)	15/15 **(100%)**
Voraxin - 3 days (14)	0/14 (**0%**)	14/14 (**100%**)	**1992** ± 194 (14)	**1111** ± 116 (14)	**54** ± 2 (14)	14/14 **(100%)**
Control (13)	0/13 (**0%**)	13/13 (**100%**)	**1952** ± 131 (13)	**1045** ± 86 (13)	**53** ± 2 (13)	13/13 **(100%)**

[a]25-28 female ticks that had been previously fed with males injected with subolesin or subolesin+voraxin dsRNAs, and did not enter the rapid phase of feeding, were placed on a fresh, tick naive rabbit and allowed to feed with uninjected males.
* and **: Level of significance with respect to the respective control is indicated: *$0.01 < p < 0.05$, **$p < 0.01$

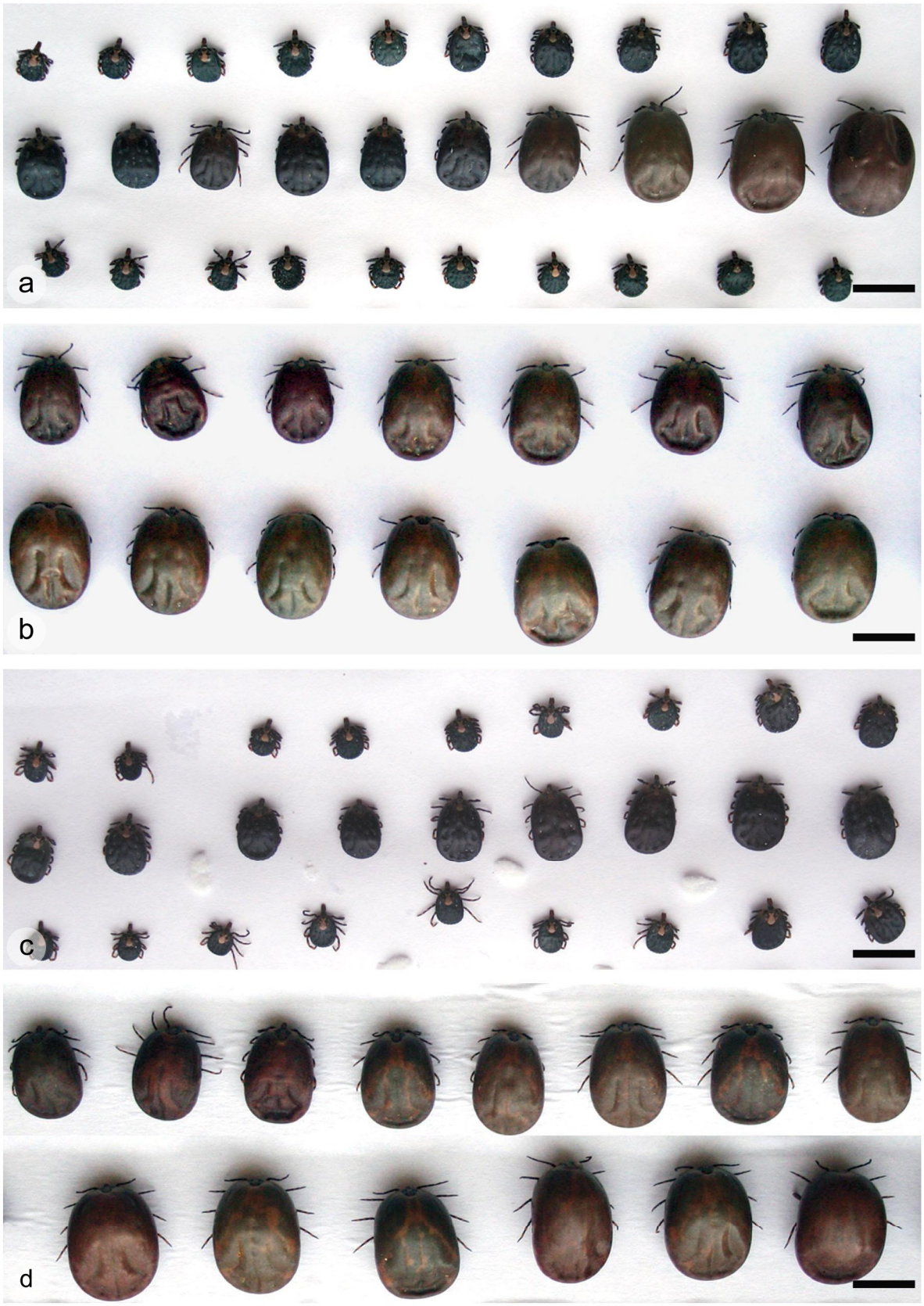

Fig 1

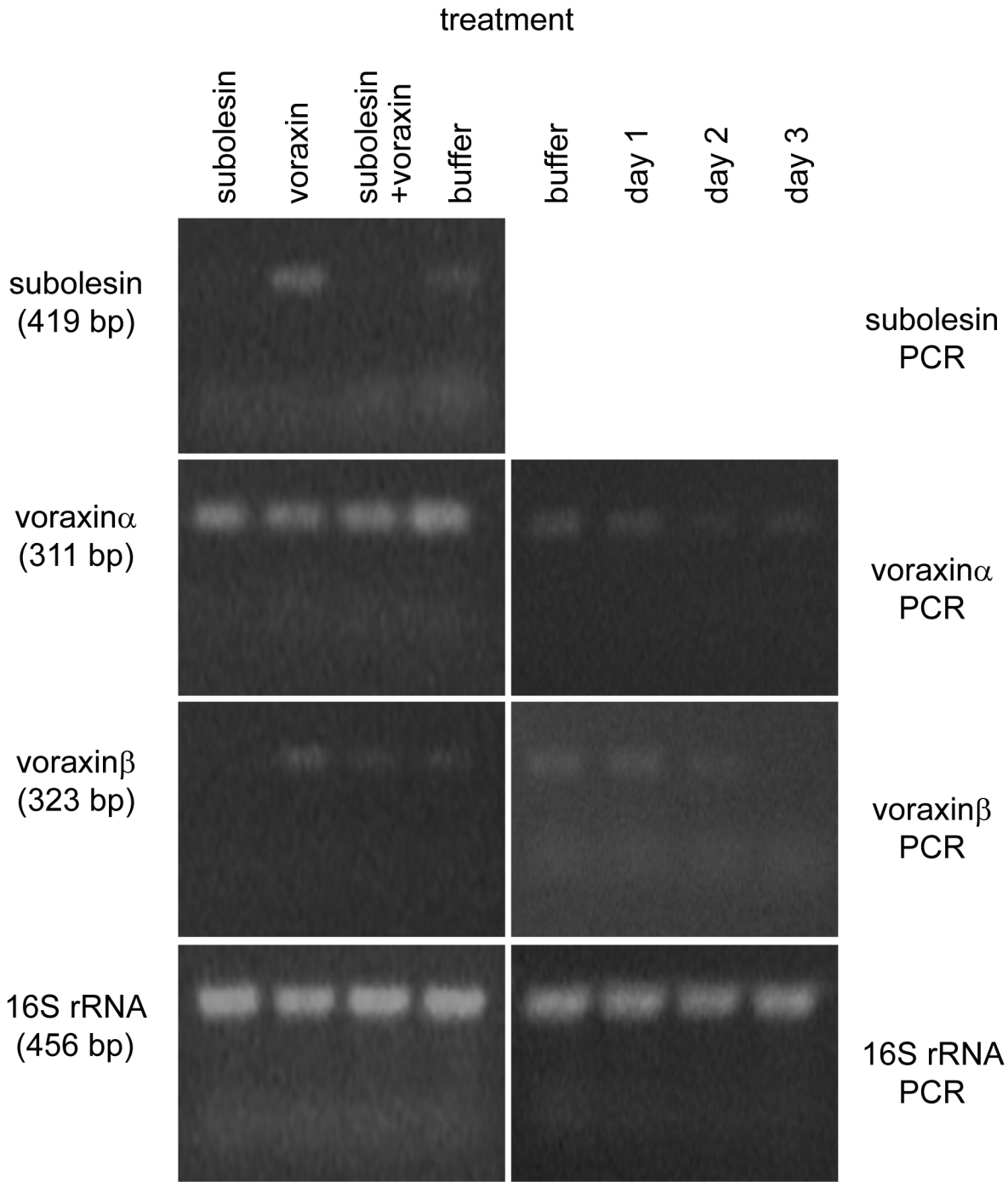
treatment
subolesin
voraxin
subolesin +voraxin
buffer
buffer
day 1
day 2
day 3
subolesin (419 bp)
voraxinα (311 bp)
voraxinβ (323 bp)
16S rRNA (456 bp)
subolesin PCR
voraxinα PCR
voraxinβ PCR
16S rRNA PCR

Fig 2

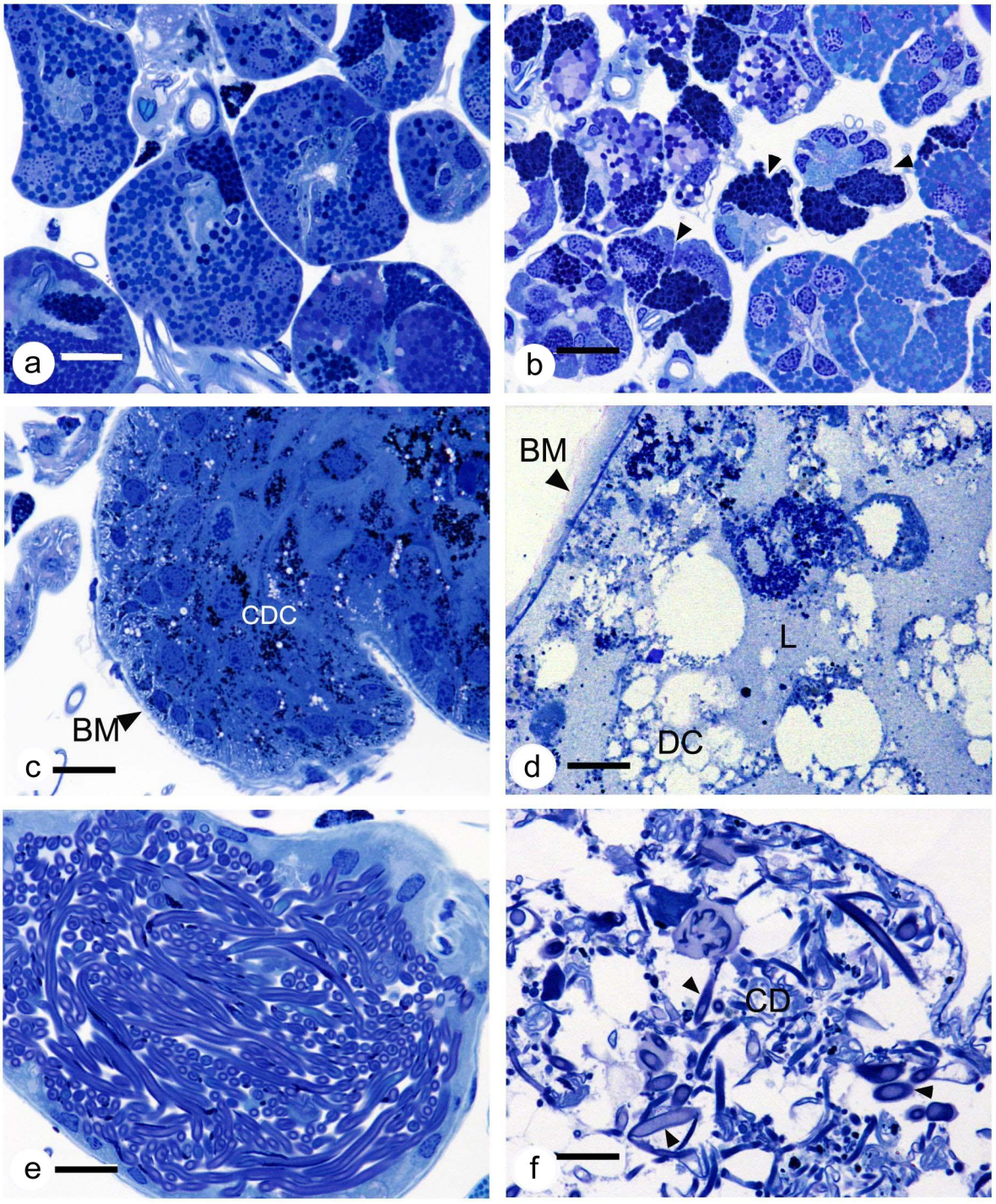

Fig 3