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Identification of two genes essential for sperm development in the male tick *Amblyomma hebraeum* Koch (Acari: Ixodidae)

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ABSTRACT

In most ticks of the family Ixodidae, gonad maturation and spermatogenesis are stimulated by the taking of a blood meal. Previous work from this laboratory identified 35 genes that are up-regulated by feeding [Weiss, B.L., Stepczynski, J.M., Wong, P., Kaufman, W.R., 2002. Identification and characterization of genes differentially expressed in the testis/vas deferens of the fed male tick, *Amblyomma hebraeum*. Insect Biochemistry and Molecular Biology 32, 785–793]. The functions of most of these genes remain unknown. We used RNA interference technology to investigate the consequences of blocking the function of 13 of these genes. Attenuation of the expression of two of these in particular, AhT/VD 8 and AhT/VD 10, correlated with deformities in the testis and abnormalities in spermiogenesis. Furthermore, most females fed in the company of these males did not engorge properly and laid many fewer eggs, most of which were infertile.

1. Introduction

Ticks are obligate hematophagous arthropods that are of great medical-veterinary importance worldwide (Jongejan and Uilenberg, 2004). The human pathogens transmitted by ticks that are currently of greatest concern are *Borrelia burgdorferi* (*sensu lato*), the etiological agent of Lyme disease, and tick borne encephalitis virus (Gern and Falco, 2000; Labuda and Nuttall, 2004).

Currently, the most widely used method for tick control is the application of chemical acaricides (George et al., 2004). The negative environmental impact of pesticides, and the eventual development of resistance in the target vector have, for decades, led to the search for alternative methods for tick control, in particular, vaccination of the host against specific tick-derived proteins (Willadsen, 2006; Sonenshine et al., 2006). For vaccination to work, however, host-derived antibodies would have to remain active while in the gut of the tick, and would have to readily cross the gut wall in active form, at least for any target antigen that was not situated in the gut epithelium itself. It is a fortunate circumstance that host-derived IgGs do meet these criteria in the case of ticks (Ackerman et al., 1981; Ben-Yakir et al., 1987; Chinzei and Minoura, 1987; Wang and Nuttall, 1994; Jasinskas et al., 2000), making vaccination a potentially excellent control strategy.

Previous work in our lab identified 35 genes that are up-regulated in the testis/vas deferens of fed vs unfed ticks (Weiss et al., 2002). Twenty-eight of these genes with complete ORFs were expressed in an insect cell expression system, and two of them, AhT/VD 9 and AhT/VD 22, were identified as the tick engorgement factor (Weiss and Kaufman, 2004). Two others, AhT/VD 16 and AhT/VD 146, showed significant homology to acylphosphatase of numerous organisms and a 9.0 kDa *Drosophila melanogaster* basic protein, respectively (Weiss et al., 2002).[2] None of the other genes showed homologies to sequences in the Genbank at the time. A more recent protein BLAST search (February 2008) reveals several more homologies that will be mentioned in the Discussion.

In this study we used RNA interference (RNAi) technology to explore the importance of 13 AhT/VD genes to testicular development and sperm function. We demonstrate that when two of these genes were inhibited by RNAi, testicular development was impaired and sperm morphology altered. Females mated with such males[3] did not fully engorge, and egg production was markedly reduced.

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[2] NB: AhT/VD 16 and 146 in Weiss et al. (2002) correspond to AhT/VD 7 and 8, respectively in Weiss and Kaufman (2004), and in this paper; AhT/VD 8 shows significant homology to ATP synthase E chain of several organisms.

[3] This study was not designed in such a way as to carry out extensive observation on male behaviour. So, in this paper, the expression "females mated with males" really means "females feeding in the company of males". In no case do we know whether the resultant infertility of the females was due to failure: of

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2. Materials and methods

2.1. Ticks and tissue preparation

The tick colony (*Amblyomma hebraeum* Koch) was reared at 26 °C, high relative humidity and in darkness. The experimental ticks ranged in age from 3 to 12 months following the adult moult. Ticks were fed on NZ white rabbits. The dorsal side of the rabbit was shaved and a foam rubber 'backpack' glued to the back with a soft latex glue (Roberts latex 8502; Bramlea, Ontario, Canada). The backpack was cut to the form of an oval with a foam partition across the center, thus forming two isolated compartments (anterior and posterior) once glued to the rabbit's back. In this way two experimental groups could be fed separately on a single rabbit.

For dissection, male ticks were fixed, ventral side down, to disposable plastic Petri dishes using a cyanoacrylate adhesive (Locktite, Mississauga, Ontario, Canada) and cooled in the refrigerator for 20 min. They were then flooded in ice-cold phosphate buffered saline (PBS: 137 mM NaCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, 2.7 mM KCl, pH 7.4), and the testis/vas deferens and accessory glands dissected out.

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.

2.2. RNA preparation and first strand cDNA synthesis

Each pair of testes was dissected out in diethyl pyrocarbonate (DEPC)-treated PBS, and kept at 4 °C in 100 μl RNAlater (Ambion, Austin, Texas, USA). RNA extraction was carried out with TRIzol reagent (Invitrogen, Burlington, Ontario, Canada) following the manufacturer's protocol. Trace DNA in the total RNA was removed with a TURBO DNA-free kit (Ambion, Austin, Texas, USA) according to the manufacturer's protocol. Total RNA was quantified by absorbance (260 nm) with a ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, USA). Samples were then stored at −70 °C. First strand cDNAs were synthesized with SuperScript III Reverse Transcriptase kit (Invitrogen, Burlington, Ontario, Canada) and Oligo(dT)12–18 Primer (Invitrogen, Burlington, Ontario, Canada) according to the manufacturer's protocol.

2.3. Synthesis of dsRNA

Thirteen pairs of primers with T7 promoter sequences specific for the 13 genes (Table 1) were synthesized by Integrated DNA Technologies (Coralville, Iowa, USA). Platinum Taq DNA Polymerase High Fidelity kit (Invitrogen, Burlington, Ontario, Canada) was used to amplify the 13 cDNAs from the synthesized first strand cDNA. The amplified cDNAs were cloned into a pGEM-T vector (Promega, Madison, Wisconsin, USA), transformed into Top 10® competent *Escherichia coli* cells (Invitrogen, Burlington, Ontario, Canada), and propagated. The sequence of each positive clone was confirmed by the Molecular Biology Service Unit of the Biological Sciences Department at the University of Alberta, using an ABI 3730 DNA analyzer (Foster City, California, USA). The templates for double-stranded RNA synthesis were amplified from the corresponding clones using the primer 5′- ATA GAA TTC TCT AGA AGC TTA ATA CGA CTC ACT ATA GGG -3′, which contains the T7 sequence to bind to both ends of the above 13 PCR products. To be used as templates for double-stranded RNA synthesis, the PCR products were purified from 1% agarose gels with a QIAquick Gel Extraction Kit (QIAGEN, Mississauga, Ontario, Canada) after electrophoresis, and quantified spectrophotometrically at 260 nm. Double-stranded RNAs of the 13 cDNAs were synthesized and purified with MEGAscript RNAi Kit (Ambion, Austin, Texas, USA), quantified spectrophotometrically at 260 nm, and kept at −70 °C until injected into the male ticks.

Table 1
Primers used for amplifying genes in this study

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
AhT/VD 1	taatacgactcactataggg atgtccgcgccagagtcc	taatacgactcactataggg ctgttgatctccaaggcggc
AhT/VD 2	taatacgactcactataggg atgcagccgcacctagatcc	taatacgactcactataggg agtttctagactgtttccagccagg
AhT/VD 3	taatacgactcactataggg atgctcctaatcgtcaccg	taatacgactcactataggg tccttttatcgcacgccc
AhT/VD 6	taatacgactcactataggg atgcagctgacccaacc	taatacgactcactataggg tcttgtgtttgcagtaaag
AhT/VD 7	taatacgactcactataggg atggtgcgcagcgcaaa	taatacgactcactataggg cagtctttttaatgctgaagtctt
AhT/VD 8	taatacgactcactataggg atggtcgaattagcccctcc	taatacgactcactataggg aagtttggaggaatcggaacg
AhT/VD 9	taatacgactcactataggg atgttgatcaccaaggacctga	taatacgactcactataggg tcgaccagtgtcaagctcgg
AhT/VD 10	taatacgactcactataggg atgagcgcgtacaaggc	taatacgactcactataggg tgggtgcggtaccactc
AhT/VD 13	taatacgactcactataggg atgctggaacagacacatctc	taatacgactcactataggg tttcctaacggttggcaag
AhT/VD 14	taatacgactcactataggg atggatctgtcctcccagg	taatacgactcactataggg aagggtgagaagttgagcag
AhT/VD 15	taatacgactcactataggg atgaggttacgcccccc	taatacgactcactataggg aaagggcctagtttggc
AhT/VD 19	taatacgactcactataggg atggtcaaatctcgaagccggc	taatacgactcactataggg tcaatgatgaatagtcctatgagtgt
AhT/VD 22	taatacgactcactataggg atggcgaaacagggactt	taatacgactcactataggg ccgcaggctcccca
16S rRNA	gacaagaagacccta	atccaacatcgaggt

2.4. Experimental protocol

For the first screen, groups of unfed male ticks (20 males per group) were injected as follows: group 1 received a mixture of all 13 dsRNAs; group 2 received AhT/VD 1, 9, 19 and 22; group 3 received AhT/VD 2, 3, 6, 10 and 13; group 4 received AhT/VD 7, 8, 14 and 15. Control ticks (group 5) were injected with RNase-free TE buffer (10 mM Tris, 1 mM EDTA, pH 7.0). After piercing the integument using the tip of a 30 g needle (usually around the lower right quadrant of the ventral surface), the dsRNA (0.5–1 μg, or about 2 × 1012 molecules of each dsRNA) was injected into the hemocoel via a 33 g needle fitted to a Hamilton® microlitre syringe. In all cases, injection volume was 5–7 μl per tick.

After dsRNA injection, the males were kept in the incubator overnight and put on a rabbit the following day. Each rabbit with a double-chambered backpack received two experimental groups. After 5 days, 12 females were put together with the males for feeding until engorgement, or for up to 21 days, after which the rabbits were terminated and all remaining ticks were removed. Engorged and removed females were weighed, and stored in the colony incubator for laying eggs. Egg masses were weighed after 50 days after detachment (by this time oviposition has essentially ceased; Friesen and Kaufman, 2002) and observed for hatching about 30 days later. The male ticks were dissected immediately after removal from the host (1) for isolation of RNA to assess the degree of RNA inhibition, (2) for histology of the testis, (3) for

(*footnote continued*)
spermiogenesis, of spermatophore formation, of spermatophore transfer, of males to attempt copulation, or of any combination of the latter.

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observing whole mounts of testis and accessory glands and (4) for examining gametes in testis squashes.

Results of the first screen suggested that groups 3 and 4 possessed genes of particular interest to this study. Thus, for the second screen, each dsRNA from groups 3 and 4 was injected into individual male ticks (11–16 males per dsRNA) to identify the specific dsRNA(s) that were responsible for the RNA interference effect observed in the first screen. As in the first screen, the injected males were kept in the colony incubator overnight and fed on a rabbit for either 5 days (AhT/VD 2, 3, 6, 10 and 13), or in one experiment (see Section 3.1) for a second 5-day period (AhT/VD 7, 8, 14 and 15), and unfed females added (one female per male). As before, females were allowed up to 21 days to engorge, after which all remaining ticks were removed. All females were weighed, and stored in the colony incubator for monitoring oviposition and hatching. As for the first screen, a few males were dissected for histology/testis squashes or for measuring the degree of RNA interference as described in Section 2.5.

2.5. Semi-quantative RT-PCR for assessing the degree of RNA interference

RNA preparation and first-strand cDNA synthesis were carried out as described above. In preparing the first-strand cDNA, equal amounts of total RNA from the treatment and control groups were used as templates, with a 1:1 M mixture of Oligo$(dT)_{12-18}$ and the reverse primer for the 16S rRNA gene, used as primers for reverse transcription, using a SuperScript III Reverse Transcriptase kit (Invitrogen, Burlington, Ontario, Canada). Serial dilutions (1/2, 1/4, 1/8, 1/16,…, 1/512) of first-strand cDNA from the treatments and their controls were used as the template for 16S rRNA gene amplification. Dilutions amplifying equivalent amounts of the 16S rRNA amplicon in both the treatment and control samples were then used as template for the amplification of AhT/VD 8 or AhT/VD 10. The primers for 16S rRNA gene are listed in Table 1. The conditions for 16S rRNA gene amplification were 94 °C (2 min), 25 cycles of 94 °C (30 s each), 50 °C (30 s), and 68 °C (1 min). The primers used for AhT/VD 8 and AhT/VD 10 were the same as described above under "Synthesis of dsRNA". The conditions for AhT/VD 8 and AhT/VD 10 were 94 °C (2 min), 24–34 cycles of 94 °C (30 s each), 60 °C (30 s), and 68 °C (1 min). PCR products from the cycle number showing the most obvious difference between each treatment and its control were recorded for the results section.

2.6. RT-PCR analysis of the transcription of AhT/VD 8 and 10 in various life stages of ticks

Total RNA was prepared from about 100 mg of the following life stages of normal ticks: eggs close to the time of hatching, fed larvae, fed nymphs, and a partially fed female adult (one that had not engorged by 21 days). The first-strand cDNA synthesis was done as described earlier. Primers for AhDV/T 8 and 10 were as described under "Synthesis of dsRNA". RT-PCR products were loaded on a 1% agarose gel containing ethidium bromide (0.5 μg/ml), electrophoresed (100 V, 30 min), and observed under UV light.

2.7. Whole mounts and squash preparations of male gonads

Intact male gonads (testes plus accessory gland), two or three samples for each treatment, were dissected out in cold PBS and placed on a glass slide in a drop of the following buffer: glycerol: 2 × PBS (1:1), and covered with a cover slip. Images were photographed under a Wild M8 dissecting microscope using a Nikon LM CCD Digital Camera. To photograph spermatids, the dissected testis (two or three samples per treatment) was squashed on the slide by applying pressure to a coverslip. Unstained spermatids were observed by differential interference contrast microscopy and photographed with a Leica DMRXA Upright Microscope fitted with an Optronics MacroFire Digital Camera. For observing the process of spermiogenesis in normal ticks, a batch of unfed male ticks was put on a rabbit, and two were removed on each of days 1–14 for monitoring spermatid development.

2.8. Histology of the testis

Dissected testes (three samples per treatment) were fixed in 4% PBS buffered paraformaldehyde, dehydrated in a series of alcohols, embedded in paraffin, sectioned at 7.5 μm and stained with Harris's hematoxylin and acidified eosin (Gurr, 1963). Images were taken with a Leica DMRXA Upright Microscope fitted with an Optronics MacroFire Digital Camera.

2.9. Statistical analysis

Statistical analysis was performed on a personal computer using Microsoft Excel software. Mean values between an experimental treatment and its corresponding control were analyzed by a two-tailed *t*-test.

3. Results

3.1. Feeding success of females mated with males injected with dsRNA

For the first screen, we injected 13 dsRNAs in one group of males and the 13 divided arbitrarily into three groups, each comprising 4–5 dsRNAs. Table 2 shows the feeding success and subsequent egg production of females mated with these males.

The mean weight of the group 1 females (398 ± 81 mg, 10) was only 20% that of the control, all of which fully engorged (2012 ± 266 mg, 8). The non-engorged group 1 females laid very few, if any, eggs; the eggs soon appeared shriveled and malformed and none of them hatched within 80 days. Control females all laid a normal egg mass (47 ± 4.7% body weight (bw), 8) and the majority of these eggs hatched. The two spontaneous engorged females of group 1 were fairly small (936 and 687 mg), but laid an egg mass (227 and 137 mg, respectively) that was normal for their low weights (Kaufman et al., 1986), and the majority of these eggs hatched.

Group 2 females (mated with males injected with AhT/VD 1, 9, 19 and 22) engorged more or less normally (1814 ± 220 mg, 9), laid normal egg masses (44 ± 4.1% bw, 9) and the majority of these eggs hatched.

Group 3 females (mated with males injected with AhT/VD 2, 3, 6, 10, 13) fed to about 45% the weight of controls (901 ± 289 mg, 10, cf. 2012 ± 266 mg, 8, $p = 0.012$), and laid an egg mass (33% bw), that was not significantly lower than the control, perhaps because of the very small sample size ($n = 3$).

Group 4 females (mated with males injected with AhT/VD 7, 8, 14 and 15), at first sight, did not seem grossly abnormal. Their feeding success was not significantly below that of the control (1576 ± 258 mg, 10 cf. 2012 ± 266 mg, 8, $p = 0.257$), and the average egg mass weight was normal. However, two of the 10 females did not engorge within 21 days. Also, a squash preparation of the testes of one of these males appeared to show some abnormality in sperm development. The sperm cells appeared longer than normal prospermia and the sperm heads were bigger.

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Table 2
Feeding success of females mated with males injected with groups of dsRNA corresponding to 13 genes up-regulated by feeding in the testis/vas deferens

	Group1[a]	Group 2[a]	Group 3[a]	Group 4[a]	Control[a]
Number of males added[b]	20	20	20	20	20
Number of females added	10	9	10	10	8
Feeding progress of females by day 21					
Number engorged	2	9	3	8	8
Number still attached	8	0	7	2	0
Female body weight (mg)±SEM	398±81	1814±220	901±289	1576±258	2012±266
p-Value (t-test) for female weight compared to the control	<0.001	0.574	0.012	0.257	–
Egg mass weight (mg)±SEM[c]	182[d]	768±113	821±528	909±163	1031±210
p-Value (t-test) for egg mass compared to the control	0.014	0.356	0.791	0.784	–
Egg mass as % body weight (±SEM)	22[e]	44±4.1	33±13.7	46±4.1	47±4.7

[a] Group 1 received all 13 dsRNAs: AhT/VD 1, 2, 3, 6, 7, 8, 9, 10, 13, 14, 15, 19, 22; group 2 received: AhT/VD 1, 9, 19, 22; group 3 received: AhT/VD 2, 3, 6, 10, 13; group 4 received: AhT/VD 7, 8, 14, 15. The controls received RNase-free TE buffer (10 mM Tris, 1mM EDTA, pH 7.0).

[b] Unfed males were injected with the indicated grouping of dsRNA, and fed for 5 days on rabbits in individual chambers. On day 6, the indicated number of unfed females was added to each chamber. Feeding progress was monitored over the next 21 days. All female ticks, including those that had not engorged and detached spontaneously by 21 days, were removed and weighed.

[c] Sample size for each egg mass is the same as the number of engorged females in that group (i.e. only the engorged females laid eggs).

[d] This mean comprises two values: 227 and 127 mg.

[e] This mean comprises two values: 24% and 20%.

Table 3
Feeding success of females mated with males injected with the individual dsRNAs from groups 3 and 4 of the first screen

	Control	Original group 3					Original group 4			
		AhT/VD 2	AhT/VD 3	AhT/VD 6	AhT/VD 10	AhT/VD 13	AhT/VD 7	AhT/VD 8	AhT/VD 14	AhT/VD 15
Number of males added	13	11	14	16	16	13	13	15	13	14
Number of females added	13	11	14	16	16	13	13	15	13	14
Feeding progress of females by day 21										
Number engorged	13	11	14	16	2	12	13	3	12	13
Number still attached	0	0	0	0	14	1	0	12	1	1
Mean female weight (mg)	1275	1736	1350	1192	440	1270	1624	524	1415	1775
SEM of female weight	122	136	150	132	92	219	134	152	287	223
p-Value (t-test) for female weight compared to control	–	0.020	0.702	0.647	<0.001	0.985	0.067	<0.001	0.660	0.063
Weight of egg mass (mg)[b]	[a]	899	523	616	476[d]	629	668	572	681	743
SEM of egg mass weight	[a]	92	88	82	–	126	93	308	165	140
p-Value (t-test) for egg mass weight compared to AhT/VD 13[c]	[a]	0.111	0.496	0.929	0.247	–	0.804	0.875	0.806	0.552
Egg mass as % body weight (±SEM)	–	50±2.8	36±3.4	49±2.0	37[e]	42±2.2	40±3.6	39±5.1	36±4.3	36±4.1

[a] These values are not available because these females were used for other purposes after recording the engorged body weight.

[b] Sample size for each egg mass is the same as the number of engorged females in that group.

[c] Because data for the controls were not available, the *p*-value here was calculated in reference to treatment AhT/VD 13, as these females were closest in mean weight to the mean weight of the controls.

[d] This mean comprises two values: 471 and 480 mg.

[e] This mean comprises two values: 40% and 34%.

We then took nine of the group 4 males and fed them for 5 days on a tick-naive rabbit and then added nine unfed females. This time only one of the nine females engorged normally (2479 mg), and laid a normal egg mass of 1331 mg. One female fed to only 510 mg, and laid very few eggs, which did not hatch. The remaining seven females fed barely at all (36±4 mg; 7). The overall feeding success of these nine females was 360±270 mg.

Because of the abnormalities just described for groups 3 and 4 females, the dsRNAs tested together in these groups were next tested individually in a second screen (Table 3). Of the group 3 females, those mated with AhT/VD 2, 3, 6 and 13 males engorged normally. However, females mated with males injected with AhT/VD 10 dsRNA fed to only 35% the weight of controls (440±92 mg, 16 vs. 1275±122 mg, 13, $p<0.001$).

Of the group 4 females, those mated with AhT/VD 7, 14 and 15 males engorged normally and laid normal egg masses, most of the eggs ultimately hatching. However, those mated with males injected with AhT/VD 8 dsRNA fed to only 41% the weight of controls (524±152 mg, 15 cf. 1275±122 mg, 13, $p<0.001$).

The females mated with AhT/VD 8 and 10 males that were still attached to the host after 21 days laid very few eggs, none of which hatched within 80 days, whereas the ones that did engorge normally laid normal egg masses, most of the eggs ultimately hatching.

Fig. 1 demonstrates directly the inhibition of AhT/VD 8 and 10 by semi-quantative RT-PCR.

3.2. Morphology/histology of the testis and progress of spermiogenesis in feeding males

Representative whole mounts of the testes and accessory gland in control and treated ticks are shown in Fig. 2. The testis of ticks injected with dsRNA of AhT/VD 8 or 10 appears significantly shrunken. The diameters of the proximal and distal part of the

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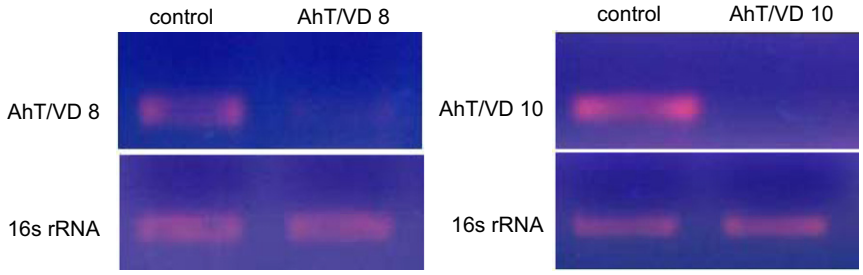

Fig. 1. Semi-quantative RT-PCR of AhT/VD 8 and AhT/VD 10 RNA in testis of fed males (see Section 2.5 for details). Although amplification of 16 sRNA was similar between control and treated ticks, amplification of AhT/VD 8 and AhT/VD 10 was much reduced compared to their respective controls.

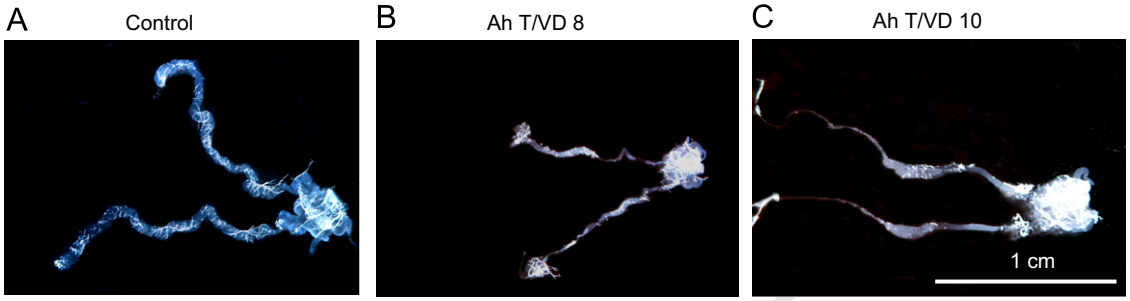

Fig. 2. Whole mounts of representative gonads from males fed for 10 days (AhT/VD 8) or 5 days (AhT/VD 10) or followed by feeding with females for a further 21 days: (A) control male (injected with TE buffer), (B) male injected with AhT/VD 8 dsRNA, (C) male injected with AhT/VD 10 dsRNA. The images shown here are representative of two or three samples per treatment.

testis were reduced by about 50–90% compared to the control specimen shown in Fig. 2A. The accessory gland also appeared shrunken; the approximate average diameter of the accessory gland (Fig. 2B) as measured along 4–5 axes was about 2.4 mm, compared to about 3.7 mm for the control (Fig. 2A). In most specimens there were noticeable differences in diameter in different regions of the testis. For example, the diameter of the distal half of the testis shown in Fig. 2C was about 90–270 μm, compared to about 620 μm for the control.

Sperm development in ixodid ticks comprises two stages: Spermatogenesis (the generation of haploid spermatids) and spermiogenesis (the transformation of spermatids to spermatozoa). In the genus *Ixodes* (prostriate ticks), feeding by the adult male is not required for generating prospermia. In all other genera of ixodid ticks (metastriate ticks), however, at least a few days of feeding is required to produce prospermia (Sonenshine, 1991).

During early spermatogenesis, the diploid spermatogonial cells undergo extensive mitoses to produce groups of spermatocytes contained in spermatocysts throughout the testes. During the late stage of spermatogenesis, meiosis results in numerous more or less spherical haploid spermatids. During the early stage of spermiogenesis, the nuclei of the spermatids move to the periphery of the cell (Fig. 3A). Further maturation involves progressive elongation of spermatids (Fig. 3B–F). During the late stage of spermiogenesis within the testis, a process of invagination from the head end results in a prospermium (Fig. 3G), the form that is packaged in the spermatophore and transferred to the female (Said et al., 1981; Oliver, 1982; Sahli et al., 1985). In all ixodid ticks, capacitation (the final stage of spermiogenesis) occurs only following spermatophore transfer to the female.

Fig. 4 shows cross-sections of treated and control testes. Fig. 4A shows the control condition. The immature spermatid diameter averaged 16.1 ±0.4 μm ($n = 10$), and most of them in this section showed oblong nuclear profiles. The mean length of ten apparently long profiles was 7.9 ±0.1 μm. Fig. 4B shows the testis from a male treated with AhT/VD 8 dsRNA. Early stage spermatids could not be found in these testes. The cross-section shown here was selected from a region in which the diameter of the testis approached that of the control condition. The spermatocytes and their nuclei appeared larger (20 ±1 and 10 ±0.2 μm, respectively; $n = 10$) than the immature spermatids shown in Fig. 4A. Regions of apparent tissue degeneration are shown in Fig. 4B. Fig. 4C shows the testis of a male treated with AhT/VD 10 dsRNA. This cross-section was also selected from a region of the testis showing the largest apparent diameter. Almost all of the nuclei in this cross-section appeared fragmented, and many clear spaces (apparent tissue degeneration) are also seen.

Squash preparations were made of the testes from the individual dsRNA treatments of groups 3 and 4 as well as controls. Fig. 5 shows squashes of control ticks at various stages of feeding. Mature prospermia were very abundant by day 9 (Fig. 5D). A similar pattern was seen in squashes of testis from males other than those treated with AhT/VD 8 and 10 dsRNA (data not shown). Fig. 6 shows a squash preparation from a male treated with AhT/VD 8 dsRNA 31 days earlier. Most of the spermatids (Fig. 6A and B) corresponded to the stages shown in Fig. 3E and F, and many of these appeared deformed in one-way or another. Some of the spermatids (Fig. 6 C) appeared to be malformed prospermia. The spermatids found in AhT/VD 10-treated males were more or less similar to those of AhT/VD 8-treated males. But the number of spermatids in treated males appeared much lower. We could find very few obvious prospermia in any of our squash preparations of ticks treated with AhT/VD 8 or AhT/VD 10 dsRNA.

3.3. RT-PCR analysis of AhT/VD 8 and 10 in various life stages

Total RNA from embryos (~50-day-old eggs laid by normal mated females), fed larvae, nymphs, and a partially fed female (one that had not engorged by 21 days) were prepared for RT-PCR amplification of AhT/VD 8 and 10 as described in Section 2.6. The results showed that both of these genes are transcribed at various

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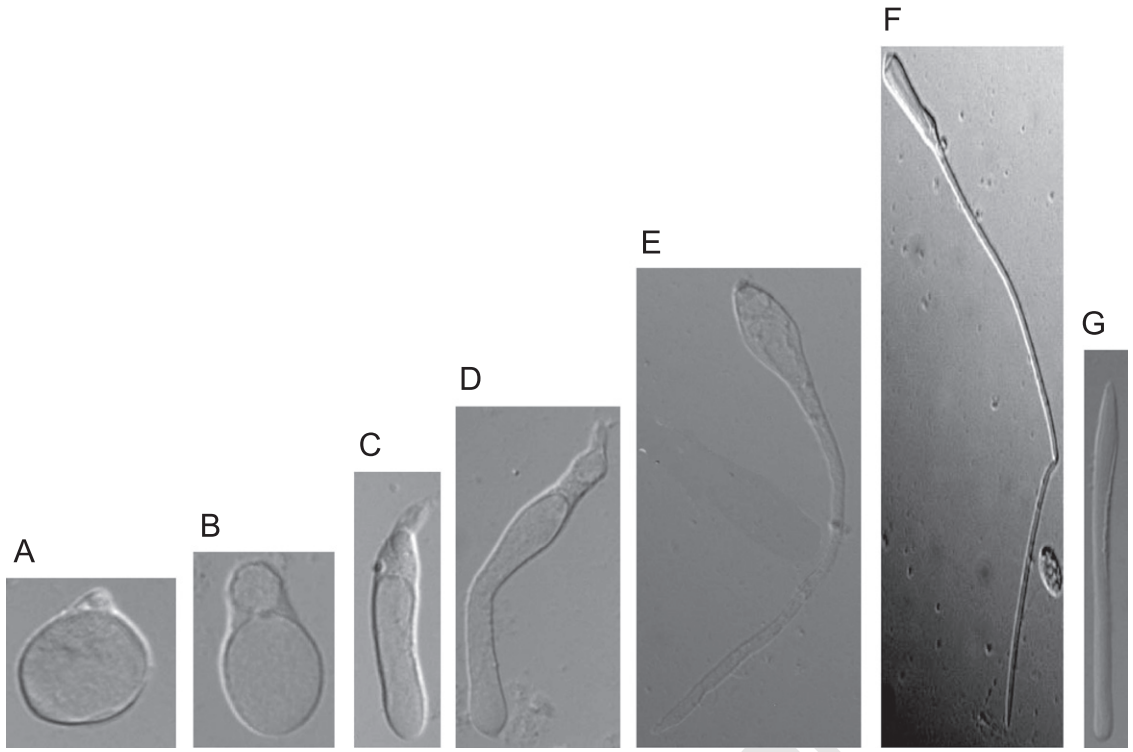

Fig. 3. Selected germ cells seen in squash preparations of testis from normal males fed for various days and viewed by differential interference contrast microscopy. In this study, germ cells reached the early spermatid stage by day 6 of feeding (A). Progressive elongation (B-F, selected from one preparation), and invagination to form the prospermium (G, selected from another preparation), occurred between 6–8 days. By day 9, almost all testes contained numerous prospermia ready for packaging in a spermatophore. The images shown here are representative of two or three samples per treatment.

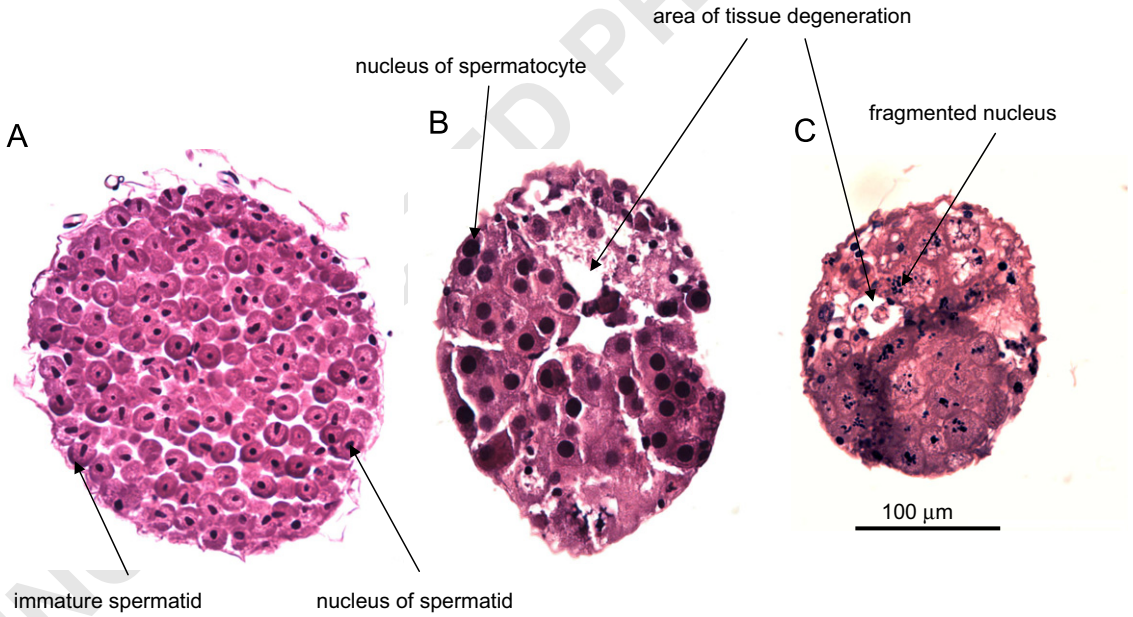

Fig. 4. Histology of the AhT/VD 8 and AhT/VD 10 knock down testis stained with Harris's hematoxylin and acidified eosin. Following prefeeding (5 or 10 days) and 21 days feeding with females (as in Fig. 2), the spermatid profiles in the control testis **(A)** appeared spherical, and were evenly distributed across the testis profile. Many nuclei have moved to the edge of the spermatid, characteristic of the early stage of spermiogenesis. In the two effective dsRNA treatments (B): AhT/VD 8, and (C): AhT/VD 10, germ cells seemed to be arrested at the spermatocyte stage. There were also many clear spaces (apparent tissue degeneration) and apparent nuclear fragmentation. The images shown here are representative of three samples per treatment. The scale bar shown is applicable to the three images.

stages of tick development (Fig. 7), suggesting that these genes control more general functions than just male-gonad development.

4. Discussion

In this study, we tested the effect of RNAi on 13 of 28 genes that are known to be up-regulated by feeding in the ixodid tick, *A.*

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hebraeum (Weiss et al., 2002); the 13 are: AhT/VD 1, 2, 3, 6, 7, 8, 9, 10, 13, 14, 15, 19, 22. Of these, AhT/VD 2, 3, 6, 13, 14, and 15 show no significant homologies in the Genbank (protein BLAST in February 2008). AhT/VD 1 shows significant homology to a speckle-type POZ protein that is widely expressed in numerous organisms. AhT/VD 7 is homologous to an acyl phosphatase that is also widely expressed. AhT/VD 8 shows significant homology to ATP synthase E-chain of *I. scapularis* and several insects. AhT/VD 9 and 22 are voraxin α and β, the two tick engorgement factor proteins that were discovered in *A. hebraeum* (Weiss and Kaufman, 2004); a protein homologous to voraxin α has also been reported in *D. variabilis* (83% identity, NCBI ref: gb/ABM92922). AhT/VD 10 shows significant homology to ubiquinol–cytochrome *C* reductase complex in numerous organisms. AhT/VD 19 shows significant homology to a thioredoxin peroxidase in numerous organisms, including the tick, *I. scapularis*. Except for the two voraxin proteins, the other genes with identified homologies appear to function in housekeeping metabolic pathways.

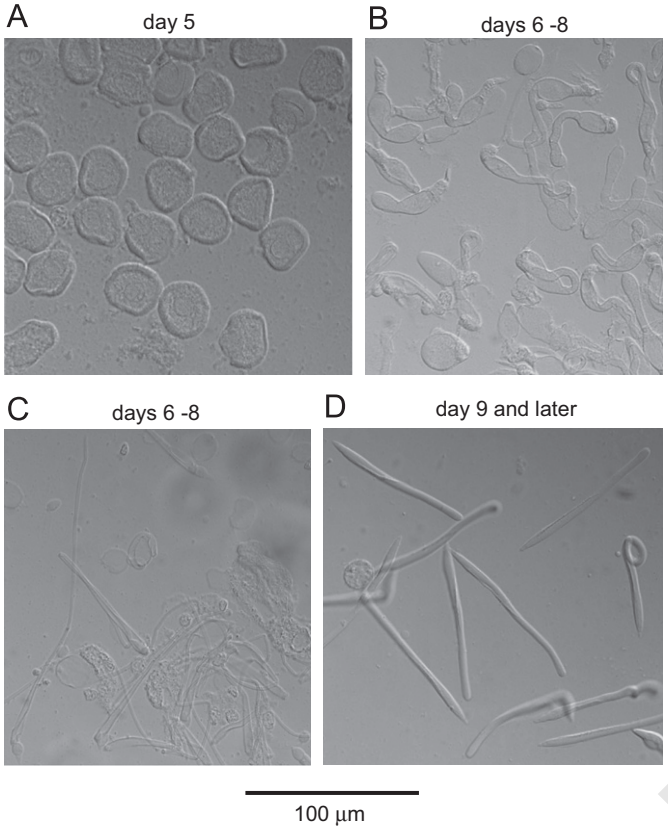

Fig. 5. Normal time course of spermiogenesis in *A. hebraeum* viewed by differential interference contrast microscopy. (A) Only early-stage spermatids were seen by day 5. (B and C) Mixed stages were seen in preparations from 6–8 days. (D) The invaginated rod-shaped prospermia, ready for insemination, were prominent in almost all males by day 9. The images shown here are representative of two or three samples per treatment.

Attenuation of the expression of either AhT/VD 8 or 10 in the tick testis by RNAi was correlated with similar histological deformities in the testes and germ cells. Moreover, most females mated with such males failed to engorge fully and laid much smaller egg masses. We cannot be sure from these data whether the reduced egg mass was due to direct effects of gene silencing or indirectly to the smaller engorged weights. However, the fact that the fewer eggs laid by females mated with dsRNA-treated males also shriveled before hatching suggests that there might be some direct effect of gene silencing in the male on the female's reproductive physiology. The female reproductive accessory gland, Gené's organ, produces a wax that protects the eggs from desiccation and from attack by soil microorganisms (Arrieta et al., 2006). Shriveling of the eggs suggests that the function of Gené's organ may have been compromised and wax secretion inhibited, although it is certainly not easy to imagine how gene knockdown in the male could directly affect an accessory gland in the female. The few females of the treatment groups that did engorge spontaneously also laid normal sized, viable egg masses. Perhaps these females mated with males in which gene knockdown was incomplete at the time of mating.

The fact that both AhT/VD 8 and 10 are transcribed in embryos, larvae, nymphs and adults (Fig. 7) indicate that they do not control processes specific to male-gonad development; the fact that the identified homologies are all to housekeeping genes also supports this. Although their attenuation in the testis was clearly

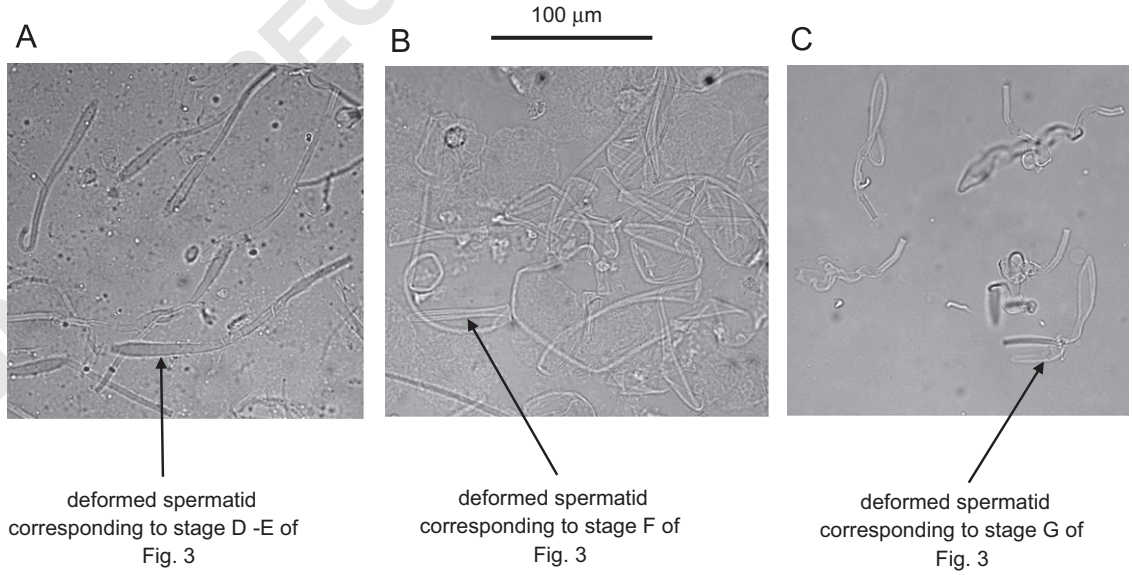

Fig. 6. Typical appearance of spermatids in AhT/VD 8 knockdown males viewed by differential interference contrast microscopy. Male ticks were dissected after 31 days of feeding (10 days prefeeding and 21 days feeding with females). While the controls always showed numerous rod-shaped prospermia (see Fig. 5D), the AhT/VD 8 dsRNA-treated testes rarely developed to this stage. Various deformities are indicated by arrows in each panel. The images shown here are representative of three samples.

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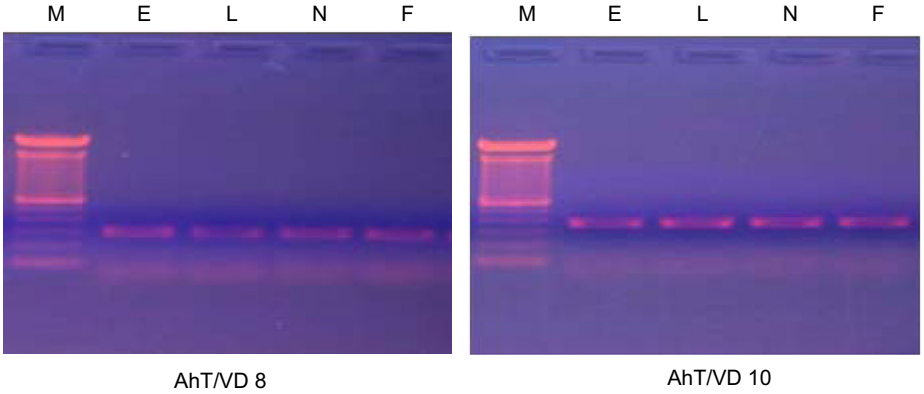

Fig. 7. RT-PCR analysis of AhT/VD 8 and 10 at various developmental stages of normal ticks. (*M*) 100 bp molecular weight ladder; (*E*) eggs close to the time of hatching; (*L*) fed larvae; (*N*) fed nymphs; (*F*) partially fed female. Thus, AhT/VD 8 and 10 messenger RNAs are not restricted to the male gonad.

correlated with a disruption of gonad development and spermiogenesis, we do not know the site or mechanism of action behind these phenotypic effects. We did not notice any other obvious physiological or behavioral changes in treated males. It would be worthwhile, however, to design experiments to enable long-term observations on mating behavior in such treated ticks.

Although we have identified only two genes up-regulated by feeding that seem to be essential for normal male-gonad development and spermiogenesis, of course we cannot conclude from this study that the other 11 genes are of little importance to male reproductive physiology. Because we tested only a single concentration of dsRNA, it is possible that higher doses would have shown an effect. However, at least some of the genes are probably associated with functions that are not directly involved with male fertility. Alternatively, RNA interference is not an all-or-none effect, and the time required for sufficient silencing to manifest itself can be highly variable (Bartlett and Davis, 2006). Our own experience in this study attests to this. Whereas the group 4 mated females had only slightly reduced engorged weights on the first feed (males fed for 5 days following injection of dsRNA; Table 1), recall that when such males were subsequently fed and mated with fresh females, the effect was considerable (Section 3.1). Thus, it is possible that at least some of the 11 genes resulting in no noticeable phenotypic effect following dsRNA injection after one feed, might have done so after a second round of feeding. Other factors also influence the apparent efficacy of gene silencing. For example, if a target gene's protein has a long biological half-life, inhibiting further synthesis by RNAi may not result in immediate or near-term loss of function (Choi et al., 2005). Knockdown efficacy also depends upon how accessible the dsRNA is to the target RNA, which may bind to other proteins and thus be protected from dsRNA (Pei and Tuschl, 2006). Finally, a targeted gene could be a member of a gene family that controls physiologically similar functions. In such a case, the effect of dsRNA would be masked by family members possessing a similar function but with a sufficient difference in nucleotide sequence so as not to have been inhibited by the dsRNA. We do not know what combination of the latter potential phenomena account for our results.

Two of the four dsRNAs injected into Group 2 males correspond to voraxin, the two engorgement factor proteins of *A. hebraeum* (AhT/VD 9 and AhT/VD 22), and yet females mated with the Group 2 males engorged normally (Table 2). This disappointing result confirms a recent study from our laboratory in which dsRNA corresponding to the two voraxin proteins likewise failed to inhibit engorgement in females mated with those males (Smith et al., 2008). In both studies, the dsRNA was injected initially into unfed males, a time at which no voraxin activity can be detected in the testis/vas deferens (Weiss et al., 2004). However, when these experiments were repeated, this time injecting voraxin dsRNA into males pre-fed for 1, 2 or 3 days, this likewise resulted in no apparent RNA interference (Smith et al., 2008). Our failure to inhibit voraxin function by RNAi remains an enigma.

The nuclear fragmentation shown in Fig. 4C appears similar to that often seen in cells undergoing apoptosis (Hacker, 2000). As mentioned above, the putative amino acid sequences of AhT/VD 8 and 10 show significant homology to ATP synthase E chain and the 14 kDa subunit of ubiquinol–cytochrome *C* reductase complex, respectively. Both these enzymes are important components of the respiratory chain of mitochondria (Arakaki et al., 2001; Braun and Schmitz, 1995). It is interesting to note, in this context, the crucial role played by mitochondria of sperm in male fertility (May-Panloup et al., 2006). A recent study on a mouse model carrying a pathogenic mitochondrial DNA deletion ('mito-mice'), demonstrates that sperm motility, and ultimately male fertility, depends on intact mitochondrial respiratory function (Nakada et al., 2006). Mitochondrial respiration defects in these mice were characterized by low sperm motility (asthenozoospermia) and low sperm count (oligospermia). Moreover, the testes of these infertile mice displayed meiotic arrest and an elevated level of apoptosis. It is certainly conceivable that the inhibition of male tick fertility observed in our study consequent to the knocking down of AhT/VD 8 or 10 might be linked to a tentative homology of these genes to those known to control key respiratory chain enzymes. The phenotypes we observed here (fragmented nuclei, apparent tissue degeneration, abnormalities in spermiogenesis and much reduced size of testis) are certainly in accord with other studies of male infertility (Nakada et al., 2006; Paasch et al., 2004).

Cytochrome *C*, a heme-containing protein, is released from mitochondria to the cytoplasm in cells undergoing apoptosis (Garrido et al., 2006; Liu et al., 1996). This could explain one interesting difference between our control and AhT/VD 8 and 10 treatments: the testes and some accessory gland lobes of these males often were noticeably violet or pink in color (data not shown). This could have been a reflection of released heme-containing proteins from mitochondria of defective sperm. In ticks, prospermia tend to be concentrated toward the distal end of the normal testis (Dumser and Oliver, 1981). Although knock down of AhT/VD 8 or 10 inhibited development of prospermia, it is worthwhile noting that it was primarily in the distal region of the testes that we noticed the pink or violet color. The pink color

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associated with the accessory gland lobes may reflect the high-energy requirement associated with production of seminal fluid.

We have shown here that the knockdown of AhT/VD 8 or 10 in male *A. hebraeum* leads to major disruption of testis development, spermiogenesis and subsequent feeding success and fertility of normal females feeding with these males. This suggests that these genes are worthy of further consideration as candidate targets, alone or in conjunction with other genes of interest (e.g. subolesin: de la Fuente et al., 2006), for an anti-tick vaccine (Willadsen, 2006).

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