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For *Insect Biochemistry and Molecular Biology*

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Sequence and the developmental and tissue-specific regulation of the first complete vitellogenin message from ticks

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Abstract

The first full-length message for vitellogenin (Vg) was sequenced from ticks. The Vg cDNA from the American dog tick, *Dermacentor variabilis* was 5744 nt in length (Genbank Accession number DQ285422) which coated for a protein of 1844 aa with a calculated molecular weight of 208 K. This protein had a 19 aa signal sequence, a single RXXR cleavage signal which predicts two subunits (49.5 and 157 K in molecular weight), and an expected lipoprotein N-terminal and carboxy von Willebrand factor type D domain. Tryptic digest MS analysis of vitellin protein confirmed the function of DQ285422 as the tick yolk protein. Apparently, vitellin in *D. variabilis* is oligomeric possibly dimeric and is comprised of a mixture of the uncleaved monomer and subunits that were predicted from the single RXXR cleavage signal. The highly conserved GL/ICG motif close to the C-terminus in insect Vg genes was different in the tick Vg message, i.e., GLCS; this variant was confirmed for another partial sequence of Vg from *Boophilus microplus*. A phylogenic analysis showed that the full length Vg from *D. variabilis* and the partial sequence from *B. microplus* was distinct from insects and Crustacea. The Vg message was not found in whole body RNA from unfed or fed males or in unfed and partially fed (virgin) females as determined by Northern blotting. The message was found in replete (mated) pre-ovipositional females, increased to higher levels in ovipositing females and was absent after egg laying was complete. The endocrine regulation of the Vg message is discussed. The tissue sources of the Vg message are both the gut and fat body. Tryptic digest MS fingerprinting suggests that a second Vg message might be present in the American dog tick, which needs further study.

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1. Introduction

Vitellogenin is a storage protein that becomes the major yolk protein of eggs. Vitellogenins are present in vertebrate and invertebrate animals, and appear to share a common ancestry (Chen et al., 1997). Vitellogenin is the initial gene product secreted into hemolymph. Once vitellogenin protein is made, the signal sequence (approximately 16 residues at the amino terminus) is cleaved and vitellogenin protein is exported from the cell. Vg protein is also split into large and small subunits and transferred to developing oocytes as vitellin, the egg storage protein (reviewed in Raikhel and Dhadialla, 1992). Vitellin also acts to store and sequester

heme in ticks (Braz et al., 1999; Logullo et al., 2002). Vitellin proteins are phosphorylated and glycosylated (Dhadialla and Raikhel, 1990; Giorgi et al., 1998).

To date, full-length vitellogenin (Vg) cDNAs have been sequenced from at least 28 insect species. Complete coding sequences of vitellogenin are available from non-insect arthropods but not ticks. Incomplete sequences have been reported from the ticks *B. microplus* (GenBank accession number U49934;Tellam et al., 2002), *Amblyomma americanum* (GenBank accession number BI27356;Bior et al., 2002) and *Dermacentor variabilis* (GenBank accession number AY885250;Thompson et al., 2005). The full-length insect cDNAs range from 5441 nt (*Apis mellifera*; Piulachs et al., 2003) to 6654 nt (*Encarsia formosa*; Donnell, 2004). Crustacean vitellogenin cDNAs range from 7782 nt (*Macrobrachium rosenbergii*; Yang et al., 2000) to 8012 nt (*Mentapenaeus ensis*; Tsang et al., 2003).

The fat body has long been accepted as the primary site of Vg RNA synthesis in the Insecta (see for example, Kokoza et al. 2001; Tufail and Tukeda, 2002 reviewed in Melo et al., 2000; Raikhel et al., 2002), although the ovaries may be a secondary producer of Vg RNA in Coleoptera (Zhai et al., 1984) and higher Diptera (Brennan et al., 1982; Isaac and Bownes, 1982). The follicle cells of *Rhodnius prolixus* synthesize Vg proteins in addition to the fat body (Melo et al., 2000). By comparison, the site of Vg RNA synthesis has been in dispute in both the Chelicerata and the Crustacea. In *Penaeus monodon* (Tseng et al., 2001) and *Macrobrachium rosenbergii* (Lee and Chang, 1999; Okuno et al., 2002), the hepatopancreas is the main vitellogenic organ. In the Crustacea *Cherax quadricarinatus* (Serrano-Pinto et al., 2004), *Penaeus semisulcatus* (Avarre et al., 2003) and *Potamon potamios* (Pateraki and Stratakis, 2000), Vg RNA is found in both

the hepatopancreas and ovary. In the shrimp, *Metapenaeus ensis*, Vg is encoded by at least two genes. One of the genes is expressed in both hepatopancreas and ovary, while the second is active in the hepatopancreas only (Tsang et al., 2003). There is evidence that *Armadillidium vulgare* (Suzuki et al., 1989) produces Vg in the ovary and the fat body, although a more recent report indicates fat body synthesis of Vg for *A. vulgare* (Okuno et al., 2000). *Crassostrea gigas* (Matsumoto et al., 2003) and *Idotea balthica basteri* (Souty and Picaud, 1981) apparently synthesize vitellogenin in the fat body alone. The site of synthesis of Vg in the hard ticks is unknown, although it has been assumed, by analogy with the Insecta to be the fat body. Chinzei and Yano (1985) identified the fat body as the source of vitellogenin in the soft tick, *Ornithodoros moubata*. However, Rosell and Coons (1992) and Coons et al. (1989), concluded that the *D. variabilis* midgut may play a role in vitellogenin production (reviewed in Sonenshine, 1991). Certainly, significant changes occur in the midgut upon ingestion of the blood meal (Agyei and Runham, 1995). Thus, there is ambiguity about the site of synthesis of the Vg in ticks.

To date, no full-length message for a Vg from ticks has been available. In the current paper, we sequenced the first Vg message and examined its expression during development and in specific tissues of the American dog tick.

2. Experimental procedures

2.1. Ticks

A pathogen-free line of the American dog tick, *D. variabilis*, was reared as described previously (Sonenshine, 1993). Adult ticks were confined within plastic capsules attached to New Zealand white rabbits (*Oryctolagus cuniculus*) and allowed to

feed and mate as required. Rearing conditions were 26±1 °C, 92±6% relative humidity and 14:10 (L:D).

2.2. Tissue dissection, egg and hemolymph collection

Ovaries, midguts and fat bodies from replete (mated, pre-ovipositing) females were dissected, washed in ice-cold phosphate-buffered saline (pH 7.0, 0.010 M NaH_2P0_4, 0.014 M Na_2HP0_4, 0.15 M NaCl) and immediately stored in RNAlater (Ambion, Austin, TX) at –80 °C until used for RNA isolation. Hemolymph was collected as described by Johns et al. (1998). Briefly, ticks were immobilized on slides, ventral side up on double-sided tape and one or two forelegs amputated with microdissecting scissors. While applying gentle pressure, clear hemolymph exuded from the opening to the body cavity was collected with a glass Drummond micropipette, transferred to an Eppendorf tube, and diluted 1:1 in PBS. Newly oviposited eggs were collected and stored overnight at –80 °C. Eggs (n=25) were rinsed once with Dulbecco's phosphate buffer (Pierce, Rockford, IL) with Tween 20 (0.05% v/v) and homogenized in 500 µl of PBST with a ceramic mortar and pestle. The homogenate was clarified by centrifugation at 960*g* at 4 °C for 10 min. Diluted hemolymph and egg homogenate were stored at −80 °C until needed for further analysis.

2.3. RNA isolation and 5' RACE

Total RNA was isolated from either whole bodies at different adult developmental stages (see results) or dissected tissues (described above) using TRI Reagent (Sigma, Saint Louis, MI) according to the manufacturer's recommendations except that RNA pellets were dissolved in water containing 10 µM aurin trichloroacetic acid to prevent degradation (Hallick et al., 1997). Samples were assayed for RNA content using a

Molecular Devices Corporation Spectromax 384 Plus plate reader (Sunnyvale, CA) and then stored at −80 °C until needed for further analysis.

The initial Vg cDNA fragment from *D. variabilis* was obtained from a cDNA library made from the fat body of replete (mated, vitellogenic) females as described in Thompson et al. (2005). To obtain the remainder of the 5' region of the Vg cDNA, 5'RACE was performed using SMART RACE (Clontech, Palo Alto, CA). First-strand Vg cDNA was synthesized from total fat body RNA (from mated (replete) pre-ovipositional eggs) in the presence of SMART IV oligonucleotide and Vg specific reverse primer (SRP). The resulting cDNA fragment was amplified using the Advantage 2 PCR Kit (Clontech). Several 5'RACE rounds were needed to obtain multiple fragments in the 5' region of the Vg cDNA, which were compiled to obtain the full Vg message. PCR amplification products were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA) and sent for sequencing in the Nucleic Acid Research Facilities in Virginia Commonthwealth University (Richmond, VA).

2.4. Northern blots

Five micrograms of each RNA sample was denatured by glyoxal treatment and separated by electrophoresis in a 1.25% agarose gel according to the phosphate protocol of Sambrook and Russell (2001). RNA was transferred by capillary action to a nylon membrane (Roche Diagnostics GmbH, Mannheim, Germany) followed by UV crosslinking according to the manufacturer's recommendations. The Vg and ribosomal probes were digoxigenin-labeled by PCR amplification. PCR conditions and reagents were according to the manufacturer's recommendations (Roche Diagnostics GmbH). Blots were pre-hybridized, hybridized and washed according to the manufacturer's

recommendation except that all elevated temperature manipulations were performed at 50 °C instead of 68 °C. Chromogenic detection was done using NBT/BCIP according to the manufacturer's recommendations.

2.5. Protein electrophoresis and digestion-mass fingerprinting

Total protein in diluted hemolymph and clarified egg homogenate was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, 1977) using bovine serum albumin (Fraction V; Fisher Scientific, Pittsburgh, PA) as a standard. The samples were then further diluted with native sample buffer (Invitrogen, Carlsbad, CA) to obtain 3.8 μg of total protein per lane for each sample to be tested. Native polyacrylamide gel electrophoresis was conducted with an Invitrogen XCell SureLock™ electrophoresis apparatus, 8–16% polyacrylamide Tris–Glycine gels, and the appropriate buffers from Invitrogen. Electrophoresis was conducted at 130 V for 4 h. Following electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 (45 ml methanol, 10 ml glacial acetic acid, 45 ml water, 0.25 g Coomassie Brilliant Blue R-250) overnight. The gels were destained with a mixture of 30% methanol and 10% acetic acid overnight and then scanned using a Hewlett Packard ScanJet 5100C. To determine the relative molecular weight of the putative Vg and Vn bands, molecular weight standards (Sigma) were run in a separate lane. Gel slices from the native gel containing the purified (putative) Vn protein from eggs were submitted to the W.M. Keck Biomolecular Research Facility at the University of Virginia (Charlottesville, VA; www.healthsystem.virginia.edu/internet/biomolec/) for tryptic digestion-mass fingerprinting as described previously (Cohen and Chait, 1997; Rao et al., 2003). After digestion with trypsin, peptides were introduced into a Thermo-Finnigan LCQ DecaXP

mass spectrometer, and the resulting spectra analyzed. The analysis does not differentiate between leucine (L) and isoleucine (I). The peptide sequences were queried by database searching using the Sequest search algorithm. Peptides that were not matched by this algorithm were interpreted manually and searched versus the EST databases using the Sequest algorithm.

Samples for SDS PAGE gels were diluted with NuPAGE LDS sample buffer (Invitrogen) to obtain 3.5 μg of total protein per lane for each sample to be tested. SDS polyacrylamide gel electrophoresis was conducted with an Invitrogen XCell SureLock™ electrophoresis apparatus, 4–12% polyacrylamide Bis-Tris gels, and the appropriate buffers from Invitrogen. Electrophoresis was conducted at 100 V for 4 h while the apparatus was in an ice bath. Following electrophoresis, the gels were stained, destained, and scanned as described before.

2.6. Sequence alignments and phylogenetic analysis of Vgs

The conceptual translation of the Vg mRNA and amino acid alignments with other Vgs were obtained using Vector NTI advance 10 (Invitrogen). Multiple sequence alignments were performed using ClustalW with gap opening penalty 10, gap extension penalty 0.05 and gap separation penalty range 8. The resulting guide tree was exported to Treeview 1.6.6 to create a radial dendrogram (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

3. Results and discussion

3.1. First full-length Vg message

A partial Vg cDNA was previously cloned in our laboratories (AY885250, Thompson et al. 2005). A forward primer designed from the 5' end of AY885250 and

5'RACE were used in the current study to obtain an additional fragment of the Vg message upstream of AY885250. This was followed by successive 5' RACE experiments using newly designed forward primers based on the new upstream fragments of Vg until the complete nt sequence of the *Dv* Vg cDNA (Genbank accession number DQ285422; Fig. 1) was obtained. In this effort, the successive 5' prime extensions contained at least a 40 nt consensus between the known and the new 5' upstream fragments. Any 5' RACE fragments, which did not match exactly in the area of overlap were not used in the assemblage of the final sequence of the Vg message. The sequence of the Vg cDNA is 5744 nt, including 19 adenine nucleotides in the poly-A tail. The 5' untranslated region is relatively short, only 42 nt. By contrast, the 3' non-coding region extends 151 nt from the stop codon (TGA) to the start of the poly-A tail.

Conceptual translation of the nucleotide sequence for Vg yields 1844 aa (Fig. 1) with a calculated (Vector NTI) molecular weight for the unmodified monomer of 208K. Analysis by SignalP indicates that the first 18 aa represent the signal sequence. Cleavage is predicted to follow this signal to yield a predicted molecular weight of 206 K. The predicted cleavage site is indicated by an arrow in Fig. 1. The molecular weight of Vg from *D. variabilis* determined by native PAGE and gel permeation chromatography was reported previously in the range of 320-486K (Sullivan et al., 1999; Gudderra et al., 2001, 2002; Thompson et al., 2005). The size of vitellin (Vn) from *Dv* by electrophoresis was in the range of 370-480K as determined by Rosell and Coons (1991) and also shown in Fig. 2 (lane 4) of this current study. Although there is considerable variation in the reports of the size of native Vg and Vn in the American dog tick, it is apparent that in all of these reports, the molecular weight exceeds that predicted from translation of the Vg

message (Fig. 1). For example, if *Dv* Vg exists as a dimer in hemolymph, the predicted molecular weight would be 412K (2 times 206K). This is in reasonable agreement with that for Vg and Vn previously reported for this species (Rosell and Coons, 1991; Sullivan et al., 1999; Gudderra et al., 2001, 2002; Thompson et al., 2005). Although we cannot confirm the exact oligomeric form of Vg and Vn in *D. variabilis*, Vg is known to exist in oligomeric forms in its native state in other Arthropods (Sappington and Raikhel, 1998); and it is apparent that the same occurs in *Dv*. Vg has not been sequenced in full from any other tick species. In *Dv*, Vg demonstrated a high content of Leu and Val (12.9 and 12.1%, respectively) followed in decreasing abundance of Pro, Lys, Thr, Glu, Tyr and Ser (7.9, 7.8, 7.5, 7.2, 7.2 and 6.6%, respectively)(Table 1). The abundance of the remaining aa was 5.8% or less.

Sappington and Raikhel (1998) noted that most insect vitellin proteins have a single cleavage site, which generates two protein subunits. The cleavage signal, RXXR, is found in *Dv* Vg at amino acids 465–468 (boxed, Fig. 1). Cleavage following this signal would yield two subunits with predicted molecular weights of 49.5K starting at the amino-terminus) and 157K ending at the carboxy-terminus. The Vg monomers of most insects are composed of one large (>150K) and one small (≤65K) subunit (reviewed in Kunkel and Nordin, 1985; Raikhel and Dhadialla, 1992; Valle, 1993) derived from the cleavage of a single precursor in the fat body (Bose and Raikhel, 1988; Dhadialla and Raikhel, 1990; Heilmann et al., 1993; Yano et al., 1994; Kageyama et al., 1994; Hiremath and Lehtoma, 1997). The predicted cleavage site for the *Dv* Vg produces similar sized subunits consistent with that found in insects. Exceptions in the insects include the Vgs of higher Hymenoptera (suborder Apocrita) (Wheeler and Kawooya, 1990; Kageyama et al.,

1994; Nose et al., 1997) and two species of whitefly (Homoptera) (Tu et al., 1997), which are not cleaved. There is evidence that some orthopteran and sawfly Vgs consist of three or four subunits (Kunkel and Nordin, 1985; della-Cioppa and Engelmann, 1987; Wyatt, 1988; Kim and Lee, 1994; Takadera et al., 1996). Vg in *Ixodes scapularis* contained 8 subunits with molecular weights from 48 to 145K (James and Oliver, 1999) while 7 subunits were reported for Vg (Sullivan et al., 1999) in *D. variabilis*. While *Dv* Vg and Vn migrates as a single protein on non-denaturing PAGE (Fig. 2, lanes 2/3 and 4, respectively), *Dv* Vn on SDS-PAGE migrated as seven major bands (data not shown). The subunits in Vn from the American dog tick were 210, 172, 157, 111, 76.2, 58.7, and 50.8 K. Two of these proteins with molecular weights of 50.9 and 157K are predicted (49.5 and 157K, respectively) based on the RXXR cleavage site at amino acids 465–468. A third 210K protein apparently arises from the uncleaved monomer which had a predicted molecular weight based on the sequence and translation of the cDNA of 206 K (after removal of the signal sequence); uncleaved Vg protein also occurs in some insects (Wheeler and Kawooya, 1990; Kageyama et al., 1994; Nose et al., 1997; Tu et al., 1997). The remaining proteins detected on SDS-PAG in *Dv* vitellin may be an artifact of the preparation and the presence of endogenous proteases as suggested by Sappington and Raikhel (1998). More studies will be needed to confirm this hypothesis. Attempts to obtain N-terminal sequence of some of the proteins resolved by SDS-PAGE were unsuccessful, suggesting that the N-terminus in some cases may be blocked.

The NCBI Conserved Domain Search identified two domains of interest in *Dv* Vg. A lipoprotein N-terminal domain, which spans amino acids 34–721 and a von Willebrand factor type D domain near the carboxy-terminus of the protein from amino

acids 1485–1655 (Fig. 1). Vitellogenins in general are predicted to contain both of these domains (Baker, 1988) in about the same positions as described for the native Vg protein of *Dv* (Fig. 1).

Among the insect Vg genes there is a highly conserved GL/ICG motif close to the C-terminus (Lee et al., 2000). Interestingly, in the crustacean sequences examined, the motif appears to be GLLG (see for example, *Macrobrachium rosenbergii* AB056458; Yang et al., 2000 *Cherax quadricarinatus* AF306784; Abdu et al., 2002 *Penaeus japonicus* AB033719; Tsutsui et al., 2000). A clotting protein from the signal crayfish (*Pacifastacus leniusculus,* AF102268; Hall et al., 1999) is homologous to vitellogenin and also contains a sequence that conforms to the insect GLCG. Interestingly, the Vg partial sequence from *B. microplus* (accession number AAA92143) and that from *D. variabilis* (Fig. 1) contain a variant sequence, GLCS, shown in italics in Fig. 1. The significance of this difference is not known.

Compared with Vg cDNA clones from other arthropods and their predicted aa sequence, *Dv* Vg aligns well with GP80 from *B. microplus*, 81% identity at the nt level and 80.2% similarity and 73.4% identity at the aa level. A phylogenetic tree was developed using the full amino acid sequence of Vgs from representative insects and Crustacea. Included in the analysis was the available sequence information from the cattle and American dog tick. In this analysis, there is clear separation of the Insect and Crustacean groups (Fig. 3) as might be expected based on their known phylogeny. The partial Vg clone from *B. microplus* and the full length protein from *D. variabilis* as might be expected based on phylogeny arose from a single node separate from the Mandibulata. Interestingly, there are 6 repeats of P(T/P)HH(K/E)(U/P) in GP80 from *B. microplus*. At

the carboxy-terminus of Vg from the American dog tick, there were 11 similar repeats of P(T/S)HH(K/E)Y with one additional repeat further upstream. None of these repeats were found in the other Vg proteins examine (Fig. 3). Vg is a heme binding protein in ticks (reviewed by Gudderra et al., 2002) but not other animals. The mechanism of association of heme with tick Vg is not known. Eventhough the P(T/P)HH(K/E)(U/P) and P(T/S)HH(K/E)Y repeats of the tick Vgs are so far exclusive to Vgs that bind heme, it appears they are not critical for heme binding. The heme binding protein, CP (discussed in more detail below), also found in *D. variabilis* hemolymph does not have these repeats. The function of the *Dv* Vg P(T/S)HH(K/E)Y repeats are currently unknown.

3.2. Tryptic digests MS confirmation of the identity of Dv Vn

When total protein from eggs was separated by native PAGE, three major proteins were detected (Fig. 2, lane 4). The protein with the lowest molecular weight (200K) is the carrier protein CP, which was previously characterized from our laboratory by Gudderra et al. (2002) and recently deduced from the sequence of the cDNA (Accession number DQ422963; protein, ABD83654). The higher molecular weight protein (??K)**(Laura, need this MW)** has not been characterized. The most abundant protein in lane 4 (Fig. 2) has an apparent molecular weight of 367.5±7.8 (1 S.E., n=3) and is presumed to be vitellin based on its abundance and size. This putative Vn protein was extracted from the gel and subjected to trypsin digest-mass finger printing. The peptides identified are shown in Table 2. Those in bold were identical to peptides identified by tryptic digest-mass finger printing of Vg from *D. variabilis* (Thompson et al., 2005). These fragments were also found in the deduced aa sequence derived from the putative *Dv* Vg message

(Fig. 1), confirming that DQ285422 codes for Vg found in the hemolymph and Vn in the eggs of the American dog tick. One peptide sequence, EPLVSTLPVHYLEELKK, was not an exact match and will be discussed in more detail later.

3.3. Developmental regulation of the Vg message and protein

In order to examine the expression of the Vg message during adult development, total RNA was isolated from unfed and fed adult male and female ticks of *D. variabilis* and subjected to Northern blot analysis using AY885250 as a probe (Fig. 4). As a control for equal loading of RNA in all lanes, an equivalent blot was probed with the ribosomal protein L10a cDNA (Accn. No. CX663255). Vg RNA was not detected in unfed or fed male *D. variabilis.* This is consistent with results from most arthropods, where Vg is not expressed in males. Unfed and part-fed, virgin *D. variabilis* females also did not express Vg RNA as would be expected but upon mating and blood feeding to repletion, Vg RNA synthesis increased dramatically (Fig. 4). In pre-ovipositional mated females there was an obvious increase in the Vg message over that of part fed females and expression increased even more in ovipositing females. The size of the Vg message was 5746±7 nt (1 S.E., n=3) as determined by Northern blotting (Fig. 4), which was in good agreement with the size of the message that was sequenced by 5' RACE, 5744 nt (Fig. 1). The intensity of hybridization with ribosomal protein L10a was nearly equivalent in all lanes, with the exception of ovipositing females, where it decreased slightly (data not shown). This is consistent with reports from mosquitoes that rprotein L10a RNA is down regulated after the blood meal (Ribeiro, 2003).

Positively correlated with the increase in Vg message after mating and blood feeding to repletion, was an increase in Vg protein in the hemolymph as determined by

native PAGE. No Vg was found in the hemolymph of part-fed, virgin females (Fig. 2, lane 1) while Vg was found in increasing amounts in pre-ovipositing (*, lane 2) and ovipositing (*, lane 3) replete (mated) females. Previous work from this laboratory has confirmed by tryptic digest-mass fingerprinting that this protein is Vg (Thompson et al., 2005). Vn from the egg is shown in lane 4 and was discussed earlier. Vg demonstrated a slightly higher molecular weight (394.7±1.2 K; 1 S.E., n=3) than Vn (compare lanes 2 and 3 with lane 4, Fig. 2) as has been shown before in insects (Raikhel and Dhadialla, 1992).

There is convincing evidence that ecdsysteroids and not juvenile hormone (JH) regulate the expression of the Vg message and deposition of the yolk protein in developing oocytes after mating and blood feeding to repletion in ticks. Neese et al. (2000) were unable to detect JH biosynthesis in *Dv* in unfed, partially fed, virgin and mated-replete females. In addition, no JH was detected in the hemolymph of mated replete females by EI GC MS. Sankhon et al. (1999) reported that ecdysteroids induced vitellogenesis in fat body from unfed adult females of the American dog tick in organ culture. In these experiments, they found significantly higher levels of Vg (by ELISA) in the incubation medium as compared to controls. These studies are corroborated by earlier studies of Taylor et al. (1997) who found that ecdysteroid injections increased hemolymph Vg concentrations in unfed *O. moubata* but only at concentrations that were toxic. Friesen and Kaufman (2002) also found that when ecdysteroids were injected into partially fed, female *A. hebraeum*, these ticks, which would otherwise be non-vitellogenic, contained Vg in their hemolymph as determined by Western blots. In addition, Thompson et al. (2005) found that injections of 20-hydroxyecdysone (20-E)

into partially fed, virgin females of *Dv* still attached to the rabbit host resulted in the appearance of the Vg message in whole body RNA extracts, the appearance of Vg in the hemolymph, an increase in ovary weight to that of replete (mated) vitellogenic females, and the deposition of yolk protein in the eggs. JH injections had no affect on egg development in these studies. This current study also shows that the appearance of the Vg message in whole body RNA during adult female development only occurs after mating and feeding to repletion and during oviposition (Fig. 4). This is correlated with the appearance of Vg in the hemolymph (Fig. 2) and is positively correlated with increased hemolymph ecdysteorid levels as has been shown by a number of investigators (Dees et al., 1984; Connat et al., 1985; Kaufman, 1991). Also, the *Dv* Vg message was not found in unfed and fed males as would be expected, since this protein is deposited in eggs. *In toto*, the developmental expression of the Vg message, the correlation of the appearance of the message with hemolymph ecdysteroid levels and the induction of Vg in partially fed, virgin females by 20-E show that ecdysteroids and not JH are responsible for the initiation of vitellogenesis in the ticks so far studied. In addition, Sonenshine (in press) has shown that the apparent uptake of Vg from the hemolymph into developing oocytes during the time of vitellogenesis requires the appearance of the Vg receptor (VgR) in the ovaries of mated, replete females. When VgR was knocked out by RNAi, replete females were unable to develop vitellogenic eggs.

3.4. Tissue sources of the Vg message

While the fat body is the location of Vg synthesis in the Insecta, Crustacea apparently can synthesize Vg in the hepatopancreas (analogous to the insect fat body), in the ovary or in both locations as previously discussed. Rosell and Coons (1992)

suggested that Vg protein may also have multiple tissue sources in ticks, i.e., the fat body and midgut. This was determined using Vg specific monoclonal antibodies and immunohistology in the American dog tick. Using AY885250 as a probe on Northern blots, we were able to also find the Vg message at high levels in total RNA extracts from fat body and gut of preovipostional (mated, replete) females of *D. variabilis* (Fig. 5). In these experiments, the message was absent in whole body RNA extracts of the negative control (part fed, virgin females) and present in whole body of pre-ovipositional (mated, replete) females (the positive control) as expected. Ribosomal protein L10a RNA levels were equivalent in all lanes, indicating equivalent loading of RNA (data not shown). The Vg RNA levels were similar in midgut and fat body, indicating nearly equivalent levels of expression of the Vg gene in these tissues. These results suggest that Vg protein is synthesized and likely secreted into the hemolymph in both the midgut and fat body of pre-ovipositing female *D. variabilis*. This is the first confirmation that Vg RNA is transcribed in the tick midgut in addition to the fat body. Interesting, Vg was also found at low levels in the ovary (Fig. 4). Whether this is fat body contamination of the ovary or actually message found in the ovary has not yet been determined. However, the ovary has been suggested as a source of Vg in the Crustacea (Avarre t al., 2003; Kung et al., 2004; Meusy and Payen, 1988). If the ovary is capable of making Vg, this Vg apparently cannot enter the egg without the ovary VgR. In knockout experiments, RNAi was shown to eliminate the VgR message and yolk uptake by developing oocytes in the American dog tick (Sonenshine et al., in press).

3.5. Possible second Vg

Most of the tryptic digest sequences shown in Table 2 were identical with portions of the conceptual translation of *Dv* Vg (shown in shadowed boxes in Fig. 1). There was 100% identity of these sequences with the conceptual translation of GP80, a partial Vg cDNA from *B. microplus*. Interestingly, the sequence "EPLVSTLPVHYLEELKK" is present in *Dv* Vg but contains mismatches (shown in bold type in Fig. 1). This sequence is repeated several times in both GP80 and *Dv* Vg at the carboxy-terminal end. The presence of these similar but not identical sequences in the protein isolated from *D. variabilis* eggs suggests the presence of more than one vitellogenin gene. This would not be unexpected, given the multiplicity of vitellogenin genes in other organisms. For example, multiple Vg genes have been identified in *C. quadricarinatus* (Serrano-Pinto et al., 2004) where two Vg cDNAs have been isolated. In the shrimp, *Mentapenaeus ensis*, there are 4 Vg genes predicted, two of which have been cloned (Tsang et al., 2003). Further studies are needed to examine this question in *D. variabilis*.

In summary, we present the first full-length sequence of the vitellogenin message from ticks. Vg has a signal peptide, an expected RXXR cleavage site, and lipoprotein and von Willebrand factor type D domains typical of other insect Vgs sequenced. Apparently Vg and Vn exists as a oligomeric protein (possibly a dimer) in tick hemolymph and eggs, respectively. Vn consist of a mixture of the native protein and two subunits produced by cleavage at the RXXR site. The Vg message was not found in unfed or fed males but was present in females after mating and blood feeding to repletion. This developmental expression of the message suggest that vitellogenesis is regulated by ecdysteroids which have been shown to occur at high levels in the hemolymh at this time and which have

been shown to induce the expression of Vg RNA in partially fed (virgin) females. There are multiple tissues sources of the Vg message, i.e., fat body and midgut, and a possible second Vg message that has not yet been sequenced.

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Figure Legends

Fig. 1. Nucleotide and amino acid sequence of vitellogenin (Vg) from the American dog tick, *D. variabilis*. The TAG stop codon is underlined. The putative poly-A addition signal is double-underlined. Peptides identified by tryptic digestion of egg vitellin are shown as shaded boxes. Amino acids in bold represent differences between the *D. variabilis* cDNA and sequences derived by tryptic digest. In particular, the sequence EYPTRHEYPTR, while a perfect match with the partial Vg sequence from *B. microplus* GP80, is repeated in Vg from *Dv* five times with four amino acid differences and twice more with 3 amino acid differences. The sequence *GLCS*, described in the text, is italicized. The fragment that was used as a probe for Northern blots is underlined with a stippled line (.....). The cleavage signal, RXXR, is indicated with an open box.

Fig. 2. Non-denaturing PAGE of tick hemolymph and eggs. Hemolymph from partly fed (virgin) females (lane 1), mated (replete) pre-ovipositional females (lane 2) and mated,

replete ovipositing females (lane 3) of *D. variabilis* was resolved by native PAGE and compared with total protein from newly oviposited eggs (lane 4). 3.8 μg of protein were loaded in each lane. The mobility of the protein molecular weight markers are indicated with arrows on the left: 132K = bovine serum albumin (BSA) dimer; 198K = BSA trimer; 443K = apoferritin (horse spleen)(Sigma). The gel was stained with Coomassie Blue. * = vitellogenin; Vn = vitellin.

Fig. 3. Phylogenetic analysis of selected insect, crustacean and Acari vitellogenins. NCBI Accession numbers: AAW78557 (*D. variabilis*), AAA92143 (*B. microplus*), CAD56944 (*A. melliphera*), AAA18221 (*A. aegypti*, VgA), AAQ92367 (*A. aegypti*, VgB), AAQ92366 (*A. aegypti*, VgC), BAA06397 (*B. mori*), BAA88075 (*P. stali* Vg1), BAA88076 (*P. stali* Vg2), BAA88077 (*P. stali* Vg3), BAD72597 (*L. maderae* Vg2), BAB19327 (*L. maderae* Vg1), AAP47155 (*S. invicta* Vg1), AAY22960 (*S. invicta* Vg2), AAY22961 (*S. invicta* Vg3), AAM48287 (*M. ensis* Vg1), AAT01139 (*M. ensis* Vg2), AAN40700 (*M. ensis* Vg3), AAG17936 (*C. quadricarinatus*), BAD98732 (*M. japonicus*), ABB89953 (*P. monodon*), AAR88442 (*F. merguiensis*), AAP76571 (*L. vannamei*), BAB69831 (*M. rosenbergii*), BAD11098 (*P. hypsinotus*), BAD05137 (*D. magna*).

Fig. 4. Northern blot of whole body, total RNA extracts from unfed and fed males, partly fed (virgin) females, replete (mated) pre-ovipositional (pre-OV) females, replete (mated) ovipositing (OV) females, and replete (mated) post-ovipositional (post-OV) females of the American dog tick, *D. variabilis*. The probe used for Northern Blots is shown in Fig. 1. The mobility of markers in base pairs is shown on the left. These results were at least duplicated with different samples.

Fig. 5. Northern blot of total RNA from whole body of partly fed (virgin) and replete (mated) pre-ovipositional (pre-OV) females compared to gut, ovary and fat body total RNA of pre-OV females of the American dog tick, *D. variabilis*. The probe used for Northern Blots is shown in Fig. 1. The mobility of markers in base pairs is shown on the left. These results were at least duplicated with different samples.

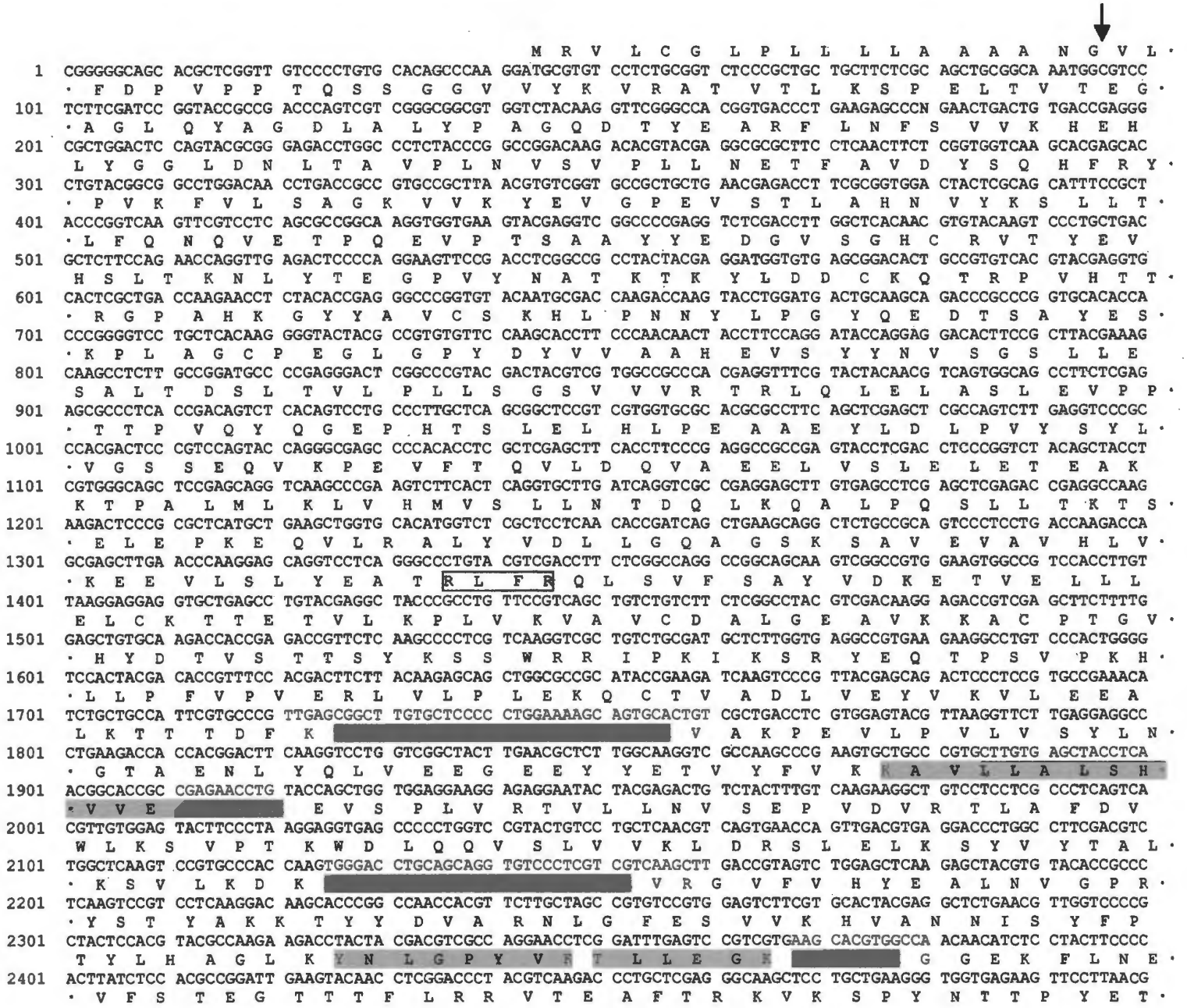
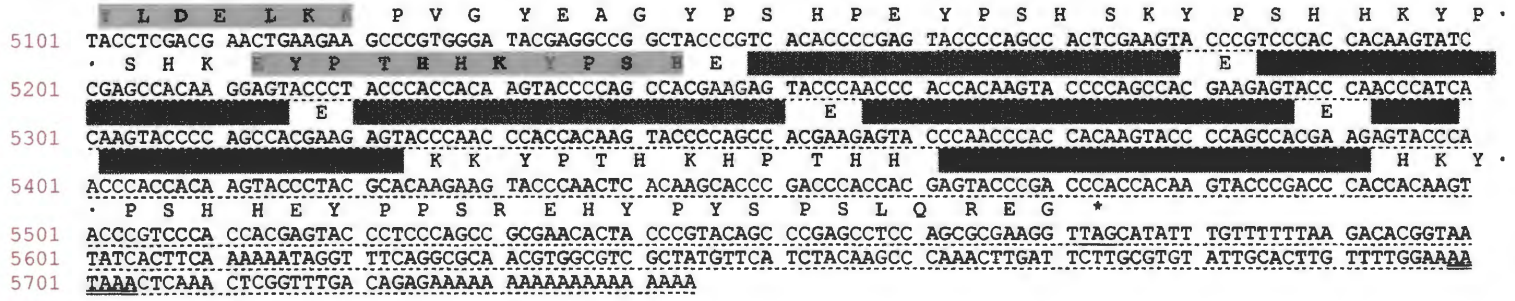


Fig. 1.

Fig 1. (*Continued*)


L D E L K P V G Y E A G Y P S H P E Y P S H S K Y P S H H K Y P
5101 TACCTCGACG AACTGAAGAA GCCCGTGGGA TACGAGGCCG GCTACCCGTC ACACCCCGAG TACCCCAGCC ACTCGAAGTA CCCGTCCCAC CACAAGTATC
S H K E
5201 CGAGCCACAA GGAGTACCCT ACCCACCACA AGTACCCCAG CCACGAAGAG TACCCAACCC ACCACAAGTA CCCCAGCCAC GAAGAGTACC CAACCCATCA
E E E
5301 CAAGTACCCC AGCCACGAAG AGTACCCAAC CCACCACAAG TACCCCAGCC ACGAAGAGTA CCCAACCCAC CACAAGTACC CCAGCCACGA AGAGTACCCA
K K Y P T H K H P T H H H K Y
5401 ACCCACCACA AGTACCCTAC GCACAAGAAG TACCCAACTC ACAAGCACCC GACCCACCAC GAGTACCCGA CCCACCACAA GTACCCGACC CACCACAAGT
P S H H E Y P P S R E H Y P Y S P S L Q R E G *
5501 ACCCGTCCCA CCACGAGTAC CCTCCCAGCC GCGAACACTA CCCGTACAGC CCGAGCCTCC AGCGCGAAGG TTAGCATATT TGTTTTTTAA GACACGGTAA
5601 TATCACTTCA AAAAATAGGT TTCAGGCGCA ACGTGGCGTC GCTATGTTCA TCTACAAGCC CAAACTTGAT TCTTGCGTGT ATTGCACTTG TTTTGGAAAA
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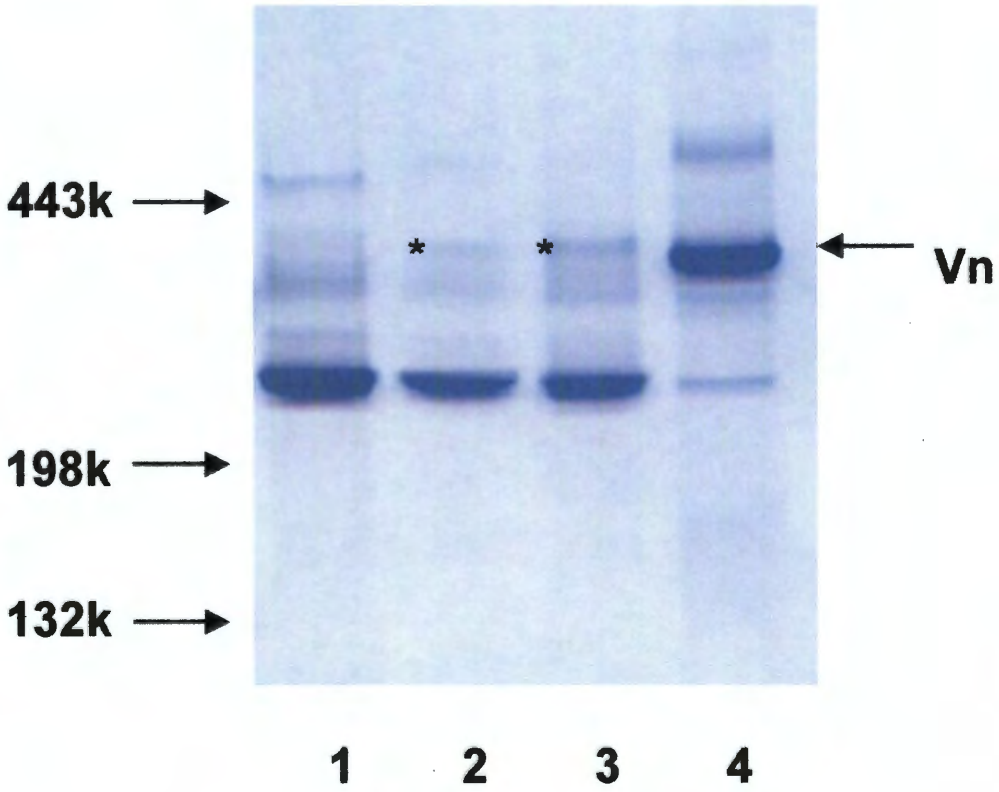

Fig 2

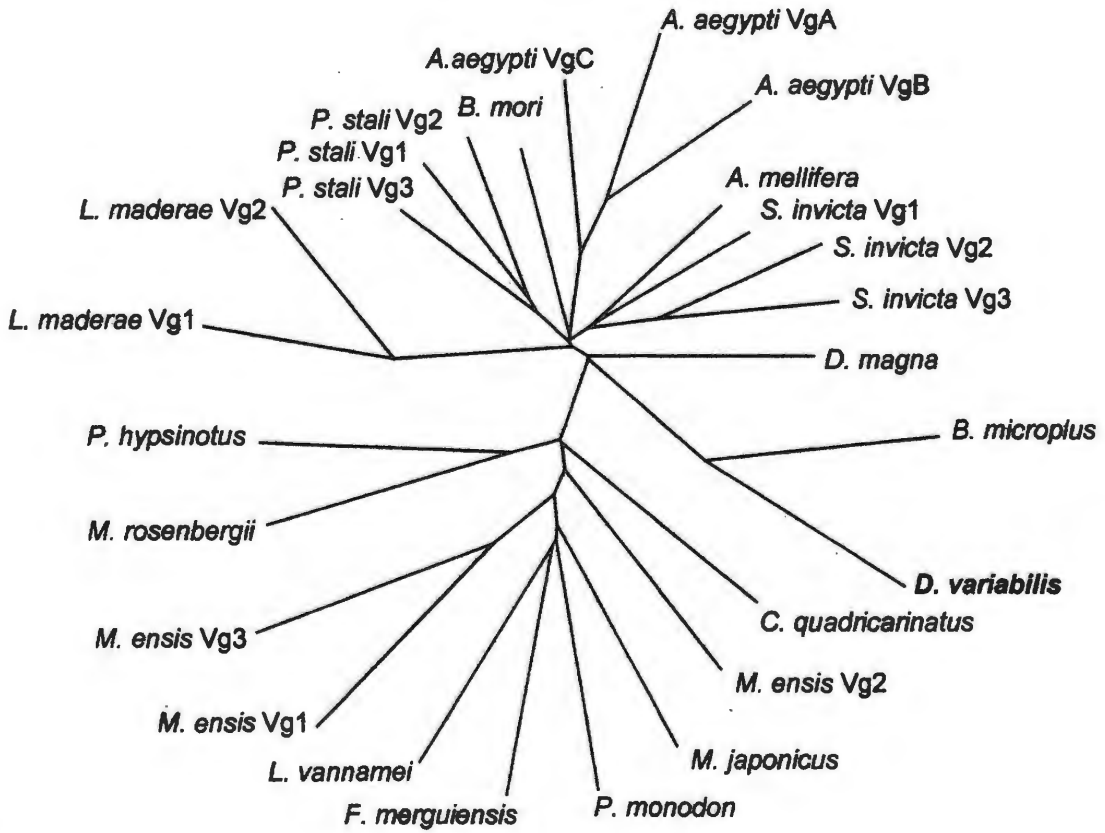
A. aegypti VgA
A.aegypti VgC
A. aegypti VgB
B. mori
P. stali Vg2
P. stali Vg1
P. stali Vg3
A. mellifera
S. invicta Vg1
L. maderae Vg2
S. invicta Vg2
S. invicta Vg3
L. maderae Vg1
D. magna
P. hypsinotus
B. microplus
M. rosenbergii
D. variabilis
C. quadricarinatus
M. ensis Vg3
M. ensis Vg2
M. ensis Vg1
M. japonicus
L. vannamei
F. merguiensis
P. monodon
0.1

Fig. 3

Fig. 4S

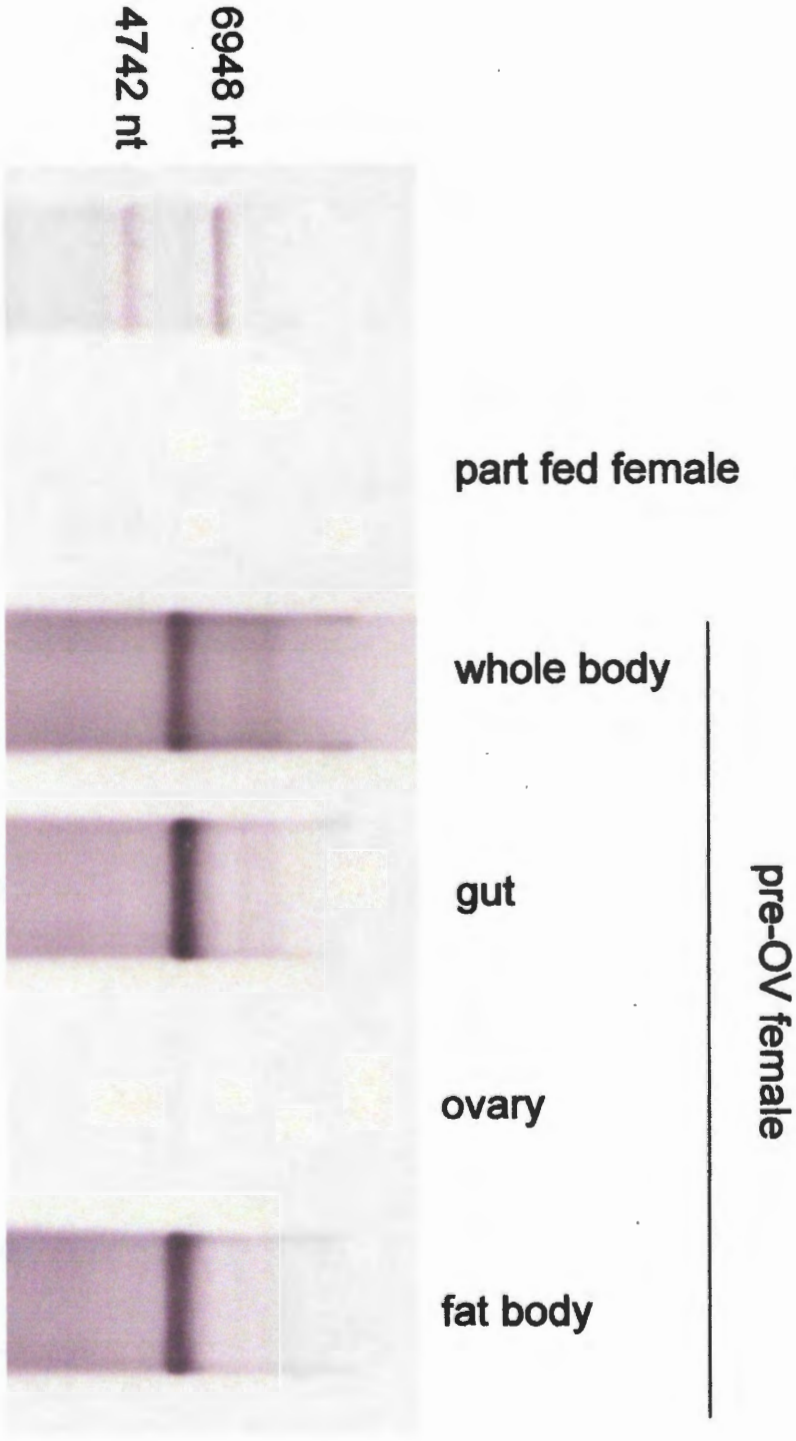
6948 nt
4742 nt
part fed female
whole body
gut
ovary
fat body
pre-OV female

Fig. 4

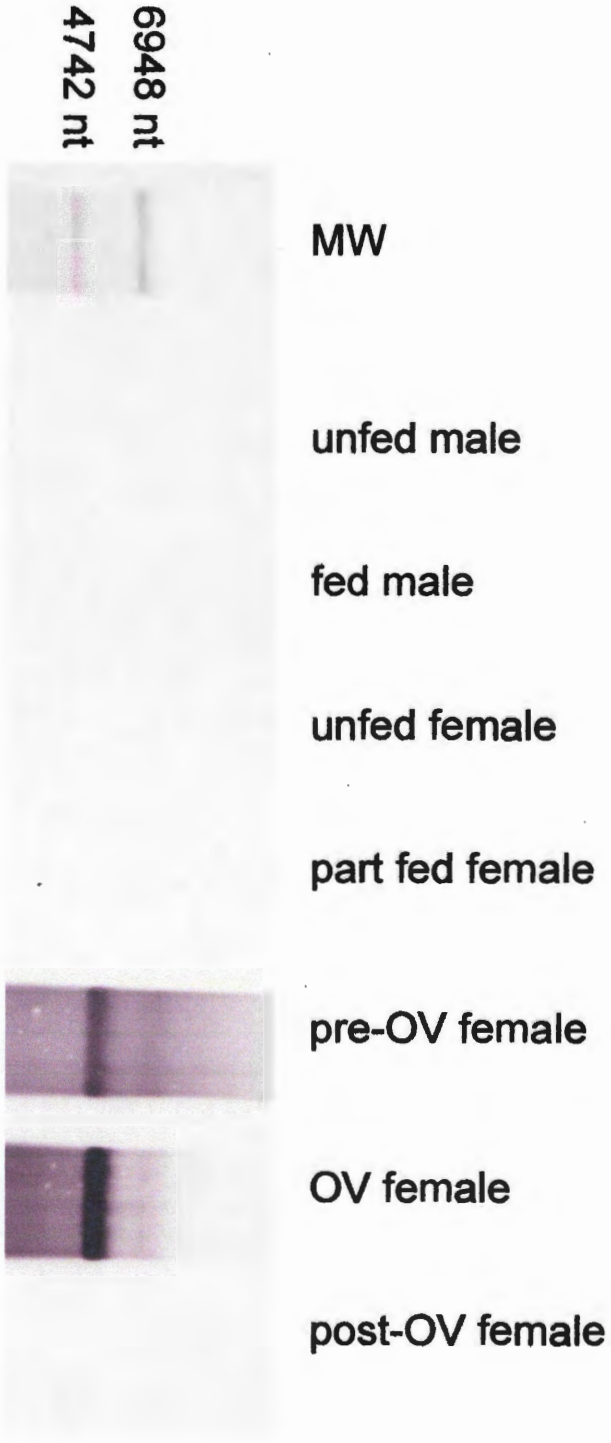
6948 nt
4742 nt
MW
unfed male
fed male
unfed female
part fed female
pre-OV female
OV female
post-OV female

Fig. 5

Table 1

Amino acid	Number	%
Ala	101	5.5
Arg	42	2.3
Asn	56	3.0
Asp	60	3.3
Cys	17	0.9
Gln	49	2.7
Glu	133	7.2
Gly	74	4.0
His	89	4.8
Ile	7	0.4
Leu	237	12.9
Lys	144	7.8
Met	8	0.4
Phe	57	3.1
Pro	145	7.9
Ser	121	6.6
Thr	138	7.5
Trp	9	0.5
Tyr	133	7.2
Val	223	12.1

Deborah, Need a title line for Table 1 on top of table.

Table 2: Peptides identified from egg vitellin.

Sequence	Mass	Charge
KAVLLALSHVVEYFPK	1814.06	3
VLVGYLNALGK	1146.69	2
YLVTSDCSAK	1143.51	2
HPANHVLASR	1101.59	3
KLIVQLGPTVVELPPPDLYK	2219.3	3
YNLGPYVK	953.509	2
HYYVDVK	923.462	2
EYPTRHEYPTR	1448.69	1
TLLEGK	660.393	1
AVLFAK	648.408	1
DKHPANHVLASR	1344.71	3
EPLVSTLPVHYLEELKK	1995.12	3
LLLK	486.365	1