

Research article

Open Access

Germ cell development in the Honeybee (*Apis mellifera*); *Vasa* and *Nanos* expression

Peter K Dearden*

Address: Laboratory for Evolution and Development, Biochemistry Department, University of Otago, P.O. Box 56, Dunedin, New Zealand

Email: Peter K Dearden* - peter.dearden@stonebow.otago.ac.nz

* Corresponding author

Published: 17 February 2006

Received: 03 October 2005

BMC Developmental Biology 2006, **6**:6 doi:10.1186/1471-213X-6-6

Accepted: 17 February 2006

This article is available from: <http://www.biomedcentral.com/1471-213X/6/6>

© 2006 Dearden; licensee BioMed Central Ltd.

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/2.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Abstract

Background: Studies of specification of germ-cells in insect embryos has indicated that in many taxa the germ cells form early in development, and their formation is associated with pole plasm, germ plasm or an organelle called the oosome. None of these morphological features associated with germ cell formation have been identified in the Honeybee *Apis mellifera*. In this study I report the cloning and expression analysis of Honeybee homologues of *vasa* and *nanos*, germ cell markers in insects and other animals.

Results: *Apis vasa* and *nanos* RNAs are present in early honeybee embryos, but the RNAs clear rapidly, without any cells expressing these germ cell markers past stage 2. These genes are then only expressed in a line of cells in the abdomen from stage 9 onwards. These cells are the developing germ cells that are moved dorsally by dorsal closure and are placed in the genital ridge.

Conclusion: This study of the expression of germ cell markers in the honeybee implies that in this species either germ cells are formed by an inductive event, late in embryogenesis, or they are formed early in development in the absence of *vasa* and *nanos* expression. This contrasts with germ cell development in other members of the Hymenoptera, Diptera and Lepidoptera.

Background

The formation and placement of primordial germ cells (PGCs) in animal embryos is of interest in developmental biology, evolutionary biology and biotechnology. In Diptera, including the well studied *Drosophila melanogaster*, PGCs form as morphologically and molecularly distinct pole cells, in the posterior of the embryo, early in development [1,2]. In insects outside the Diptera PGCs can develop in regions of pole or germ plasm, a distinct form of cytoplasm in early eggs [3], or are associated with the oosome [4-6] a specialised subcellular structure that appears to define PGC fate early in embryogenesis. These specialised structures and/or cytoplasm are widely distrib-

uted amongst insects but are not found in all insect embryos [1,7].

In the honeybee (*Apis mellifera*), little is known about the formation of PGCs in the embryo. In late embryonic and larval stages PGCs have been identified in the genital ridge situated near the dorsal surface of the abdomen where the gonads develop [8]. Previous authors have stated that there is no evidence for PGCs arising early in development [8] as is seen in other insects.

In other insects PGC placement and development has been studied using molecular markers of germ cell fate

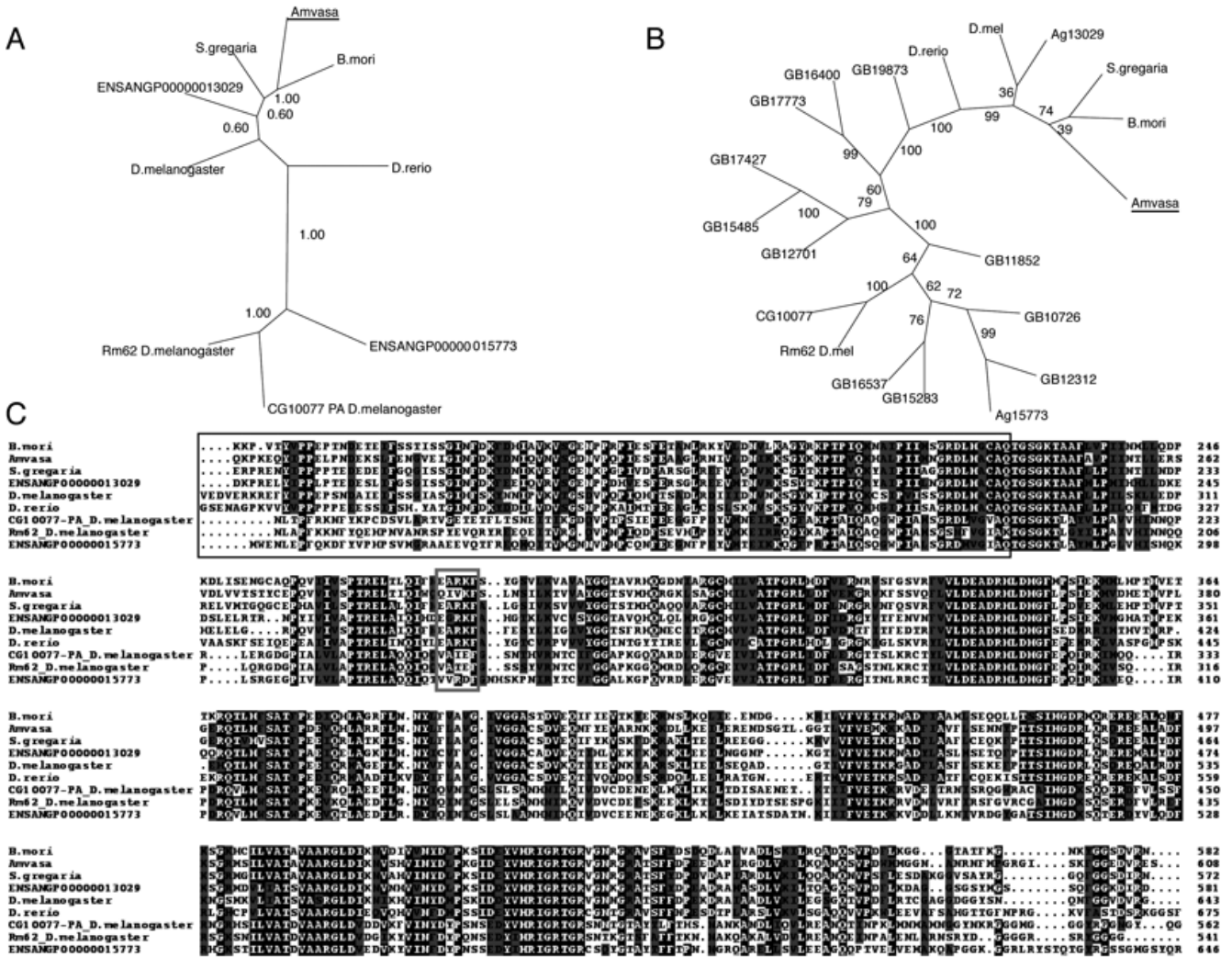


Figure 1
Analysis of *Apis vasa*. A) Bayesian phylogeny of *vasa* protein sequences and Rm62-like DEAD box helicases from insects (and *Danio*). The predicted *Apis mellifera* *vasa* protein sequence clusters with other insect *vasa* proteins to the exclusion of Rm62 DEAD box helicases. ENSANGP00000013029 and ENSANGP00000015773 are *Anopheles* predicted protein sequences. This phylogram was derived from a multiple alignment shown in C. B) Neighbour joining phylogeny of *vasa* protein sequences and honeybee predicted DEAD-box containing proteins. *Amvasa* is the only *Apis* DEAD-box containing gene to cluster within the *vasa* clade. C) Multiple alignment of the most conserved domains of *Vasa* predicted proteins. The region boxed in red is the EXRKF motif that is not conserved in Honeybee *vasa*. The region boxed in black is the predicted protein encoded by the 3' end of the probe used for in-situ hybridisation.

[7,9,10]. Two genes, *vasa* and *nanos*, have proved useful markers for PGCs in a broad range of species.

The *vasa* gene encodes a DEAD box RNA helicase that is expressed in the PGCs of all major groups of animals examined [11-17]. In mice, *vasa* related genes are expressed in PGCs and are required for PGC development [18]. In *Drosophila* *vasa* is required for multiple processes in the development and maintenance of PGCs (reviewed in [19]) including the localisation of *nanos* RNA [20].

Nanos genes encode zinc finger transcription factors that have been shown to be expressed in the PGCs of Diptera [21-23], *Caenorhabditis elegans* [24], Cnidarians [25,26], Leech [27,28], mice [29] and humans [30]. In *Drosophila* *nanos* is required for PGC migration and fate [21] and acts to repress somatic cell fate in PGCs by repressing differentiation [31,32]. In Zebrafish, *nanos* is required for PGC migration and survival [33]. Mice have three *nanos*-like genes, two of which are expressed in PGCs and required for their maintenance [29].

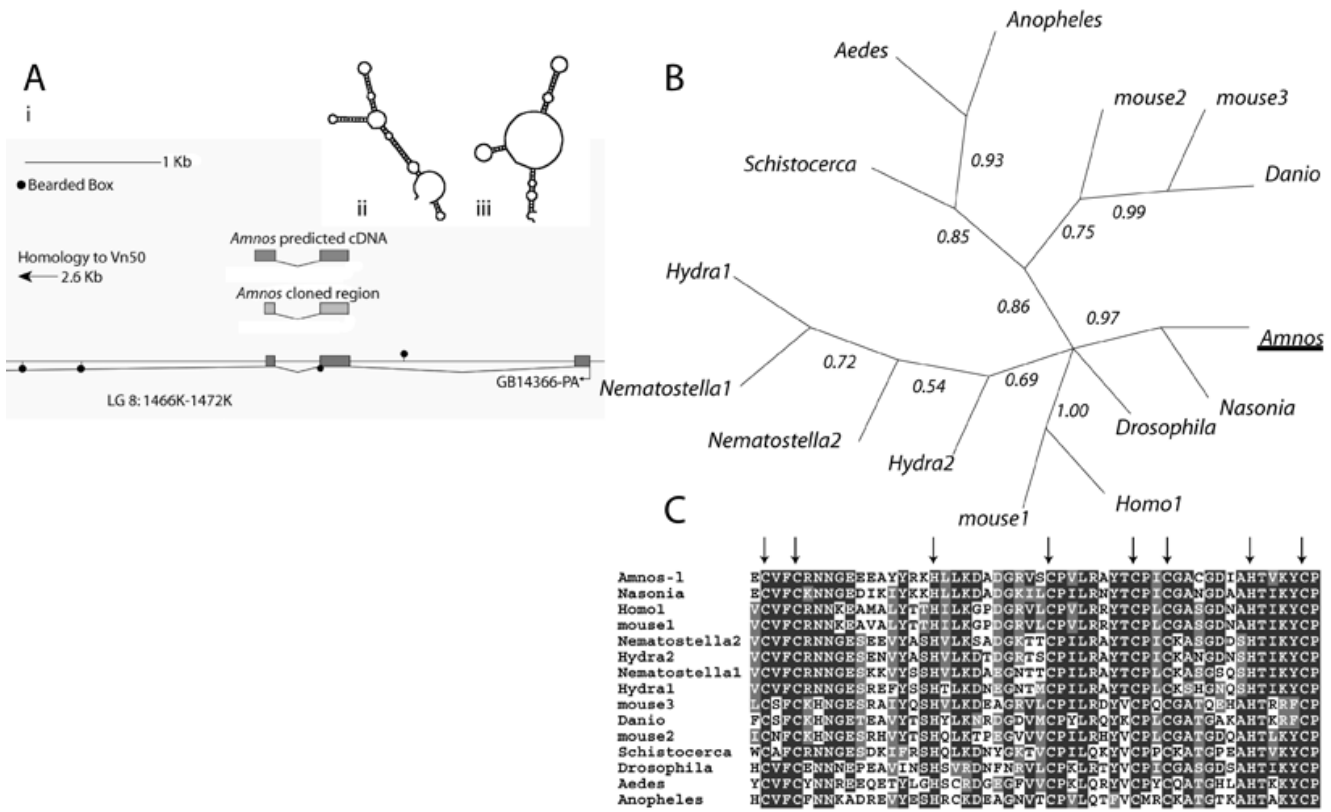


Figure 2

Analysis of *Apis nanos*. A) i) Map of the genomic region that contains the highest similarity to *nanos* sequences in the bee genome. The map indicates the regions of similarity to *nanos* genes, the cDNA region cloned and the position of putative bearded boxes associated with *nanos* translation regulation in *Drosophila*. ii and iii) RNA structures of the first 130 bases of the *Drosophila nanos* (ii) and *Amnos* (iii) 3'UTRs predicted by MFOLD [42]. B) Bayesian phylogeny of *nanos* protein sequences derived from the alignment shown in D. The predicted *Apis mellifera nanos* protein sequence (*Amnos*, underlined) clusters with *Drosophila* and *Nasonia nanos* sequences with high posterior probability. C) Alignment of the most conserved regions of *nanos* protein sequences from metazoa.

Vasa expression, assayed with in-situ hybridisation or antibodies, has been used most generally as a PGCs marker in arthropod embryos. Using both techniques, PGC specification has been studied in the orthopteran *Schistocerca gregaria*. In this species PGCs form at the dorsal boundaries of the germband in the abdomen and migrate dorsally to the gonads [7]. *Vasa* expression has also been examined in the lepidopteran *Bombyx mori* [9]. In this species, *vasa* RNA is first expressed in the presumptive embryo and then comes to be located in cells at the posterior of the germ band. In later stages these *vasa* positive cells populate the abdomen in regions consistent with the forming gonads. In non insect arthropods, *vasa* expression has also been examined in the two-spotted spider mite (*Tetranychus urticae*) [11], and the crustaceans *Daphnia* [12] and *Parhayle* [13]. In *Tetranychus*, *vasa* RNA marks a population of cells underlying the cellular blastoderm that migrate into the posterior of the embryo and

are incorporated into the gonad. In *Daphnia*, *vasa* protein is localised in a subcellular compartment in 8 cell stage embryos and is partitioned into a single blastomere at the 16-cell stage. This *vasa* positive cell is the progenitor for the germ line in this species [12]. In *Parhayle*, *vasa* protein expression becomes apparent at the 8-cell stage in the g-micromere. Manipulation of the embryo, however, indicates that localised determinants for PGC development exist at the 2 cell stage [13].

In Hymenoptera, the insect order containing the honeybee, *vasa* expression has been examined only in the polyembryonic parasitic wasp *Copidosoma floridanum*. In this species, an organelle, the oosome, stains for *vasa* RNA and is segregated into cells that then express *vasa* RNA and protein and form PGCs. Inheritance of PGCs appears to regulate both larval caste and axis formation [10,34]. Oosomes that specify germ-cell fate are found in many

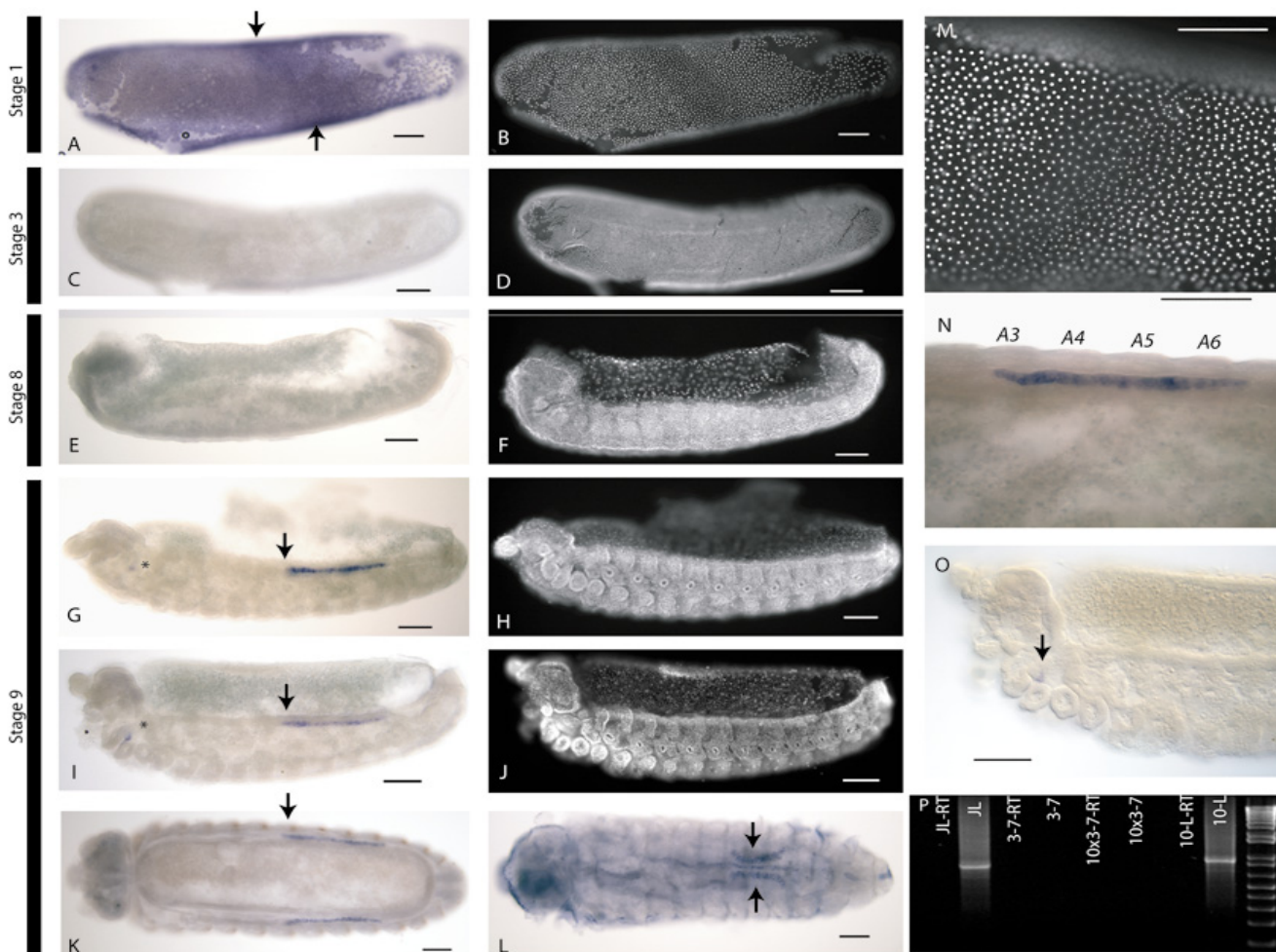


Figure 3
Expression of *Amvasa* in *Apis mellifera* embryos. Scale bars indicate 100 micrometers. A, C, E, G, and K, Brightfield images of embryos hybridised with probes against *Amvasa*, Lateral view. B, D, F, H, J, Fluorescent micrographs of the same embryos stained using the nuclear stain DAPI. A and B) A stage 1 embryo stained (blue) for *Amvasa* RNA. Expression is present in the cytoplasm of all energids. The dark band running across the embryo (arrows) is due to mitosis occurring in that region (see B and magnified in M). C-F) embryos of stage 3 (C and D) and stage 8 (E and F) showing no expression of *Amvasa* RNA. G-J) embryos of stage 9 stained for *Amvasa* RNA. Expression can be seen in a line of cells starting in the 3rd abdominal segment (arrow in G and I) and stretching back to the 6th abdominal segment. The line of cells expressing *Amvasa* (magnified in N) lies under the epidermis and is just ventral to the boundary between the germband and the extra-embryonic membranes. A small group of cells expressing *Amvasa* can also be seen in the mandibular segment (asterisks in G and I). K) View of the dorsal surface of a stage 9 embryo stained for both *Amvasa* RNA (blue) and engrailed-like proteins (brown). Arrow marks the third abdominal segment. L) View of the dorsal surface of a just-hatched honeybee larva stained for *Amvasa* RNA. Expression can be seen in two crescents of cells near the midline of the larva (arrows) in the approximate position of the future gonads. The background in this larva is due to the forming cuticle. M) Magnification of the stage 1 embryo shown in A and B stained with the nuclear dye DAPI showing a region of mitoses in the embryo. N) Magnification of the *Amvasa* expressing cells of a stage 9 embryo, dorsal view, segments marked. O) Magnification of the anterior region of a stage 9 embryo stained for *Amvasa* RNA. A group of cells in the mandibular segment are weakly expressing *Amvasa* RNA (arrow). P) RT-PCR analysis of *Amvasa* expression. RT-PCR was carried out on RNA from just-laid eggs (JL), eggs between stages 3 and 7 (3-7) and eggs older than stage 10 (10-L). Each RT-PCR experimental lane lies next to a control reaction where amplification was from RNA treated as for the experimental lanes but without the addition of reverse transcriptase in the first stand synthesis. This controls for genomic DNA contamination of the cDNA. These lanes are labelled with -RT. RT-PCR was also attempted from ten times more cDNA from stages 3-7 in an attempt to detect low level expression (10 × 3-7) Successful amplification of *Amvasa* gives a 916 bp fragment. Amplification is seen only in cDNA from just laid embryos and stages 10 and above.

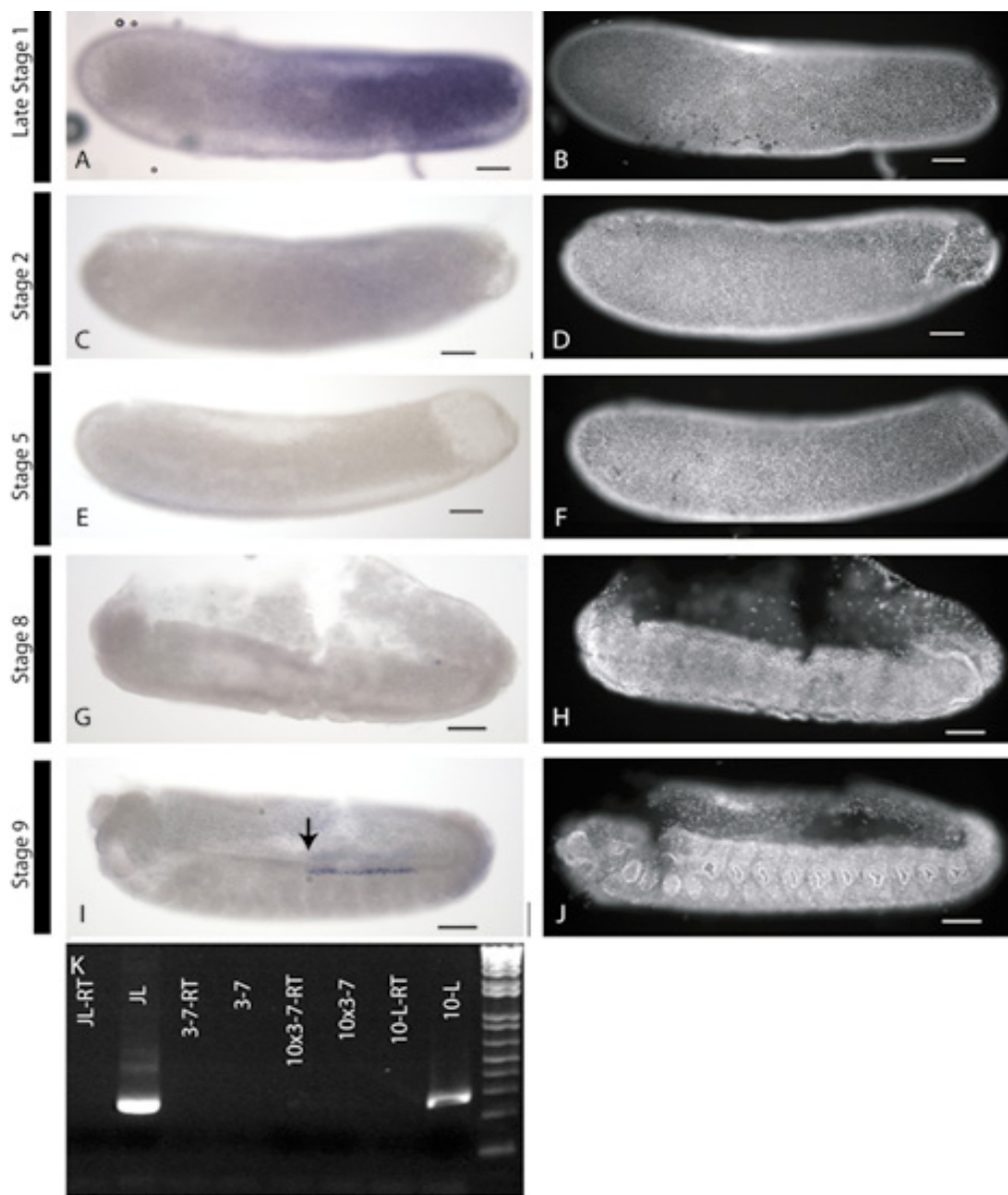


Figure 4

Expression of *Amnos* in *Apis mellifera* embryos. Scale bars denote 100 micrometers. A, C, E, G and I, Brightfield images of embryos hybridised with probes against *Amnos*. Lateral view. B, D, F, H, J, Fluorescent micrographs of the same embryos stained using the nuclear stain DAPI. A and B) A late stage 1 embryo stained for *Amnos* RNA. *Amnos* RNA is present in a gradient with highest concentrations at the posterior pole. C and D) a stage 2 embryo stained for *Amnos* RNA. Faint staining is present in a similar pattern to that in A. *Amnos* RNA expression fades very fast in stage 2 embryos. E-H) Stage 5 (E and F) and stage 8 (G and H) embryos stained for *Amnos*. No expression is seen. I and J) Stage 9 embryo stained for *Amnos* RNA, expression is seen in a line of cells starting in the third abdominal segment (arrow in I) and stretching to the 6th abdominal segment. The *Amnos* expressing cells underlie the ectoderm of the embryo and lie just ventral to the border between the germband and the extraembryonic membranes. K) RT-PCR analysis of *Amnos* expression. RT-PCR was carried out on RNA from just-laid eggs (JL), eggs between stages 3 and 7 (3-7) and eggs older than stage 10 (10-L). Each RT-PCR experimental lane lies next to a control reaction where amplification was from RNA treated as for the experimental lanes but without the addition of reverse transcriptase in the first stand synthesis. This controls for genomic DNA contamination of the cDNA. These lanes are labelled with -RT. RT-PCR was also attempted from ten times more cDNA from stages 3-7 in an attempt to detect low level expression (10 × 3-7) Successful amplification of *Amnos* gives a 257 bp fragment. Amplification is seen in cDNA from just laid embryos (JL), and cDNA stages 10 and above.

hymenopteran species [4,6] but are not present in Honeybee ovaries or embryos [35].

Nanos genes have a role in specifying posterior regions of insect embryos [22,23,36-38]. In *Drosophila*, *nanos* RNA is localised to posterior regions of the embryo, and *nanos* translation is repressed in the rest of the embryo by the binding of *smaug* protein to a RNA secondary structure in the 3'UTR of the *nanos* mRNA [39]. *Nanos* protein then regulates Hunchback (Hb) translation by recruiting a cofactor, *pumilio*, that binds a 'nanos response element' in the 3'UTR of the Hunchback mRNA, thus restricting Hb protein expression to the anterior of the embryo [37]. This translation repression activity in the posterior of insect embryos appears to be conserved in the orthopteran *Schistocerca americana* [36] where *nanos* RNA is also posteriorly localised. Posteriorly localised *nanos* expression is also found in mosquito embryos [22].

In this paper I have cloned cDNA fragments of Honeybee *vasa* and *nanos* and used in-situ hybridisation on embryos and ovaries to determine if honeybee PGCs are specified early in development, like many holometabolous insect embryos, or if they form late in development from an inductive event.

Results

Identification of homologues of *nanos* and *vasa* in honeybees

Reciprocal blast searches [40] of the honeybee and *Drosophila melanogaster* genomes with *Drosophila vasa* and *nanos* sequences identified a single region of the honeybee genome with homology to *vasa*, and a single region with homology to *nanos*. Gene predictions corresponding to each of these regions were collected and analysed.

A predicted transcript (GB14804-PA, DQ288391) located on group 1.64 has high homology to *Drosophila vasa*. I term this transcript *Amvasa*. The transcript is predicted to encode a protein containing all of the 8 domains conserved in DEAD box helicases. The GIVGXA motif, conserved in insect *vasas*, is also conserved but the EXRKF domain conserved among *Vasa* and PL10 proteins is not present. The function of this motif is not known. The full length of this transcript was cloned from cDNA.

Phylogenetic analysis of the *Amvasa* predicted protein sequence indicates that it clusters with *Drosophila*, *Bombyx* and *Schistocerca vasa* proteins as well as a predicted vasa-like dead box helicase from *Anopheles* (ENSANGP00000013029) (Figure 1A). Related non-*vasa* DEAD-box helicases form a separate clade in this analysis, indicating that the *Amvasa* is a homologue of *Drosophila vasa* and not a related DEAD-box helicase. The lack of the *vasa* specific EXRKF motif is unusual for an insect *vasa*

protein. To ensure that the gene identified is the orthologue of *vasa* from the Honeybee, the Honeybee genome was searched for regions encoding DEAD box helicase proteins. All of the DEAD-box encoding regions have been identified in the GLEAN3 official gene prediction set. Neighbour joining phylogenetic analysis of a multiple alignment of the predicted proteins encoded by these sequences and other insect and vertebrate DEAD-box helicase proteins indicates that the *Amvasa* gene is the closest homologue of *vasa* in the Honeybee genome (Figure 1B)

The sequence with highest homology to *Drosophila nanos* in the Honeybee genome is included in a predicted transcript (GB14366-PA) that is annotated as similar to venom protein Vn50 (Located on group 8.7). Analysis of this transcript indicates that homology to *nanos* is restricted to two upstream exons almost 7 KB from the exons of the predicted transcript with homology to venom protein Vn50 (Figure 2A). Just downstream of the second exon corresponding to *nanos* is a gap in the genome sequence that may have caused an error with the gene prediction software. I have sequenced across this gap and identified an in-frame stop codon downstream of the second exon. Primers designed to amplify full-length *nanos* from cDNA indicate that the gene prediction is correct in this area. I designate the gene from which this sequence derives *Amnos*. This sequence is deposited in Genbank as [DQ288392](http://www.ncbi.nlm.nih.gov/nuccore/DQ288392).

Multiple alignments of predicted proteins from *nanos* relatives indicate that the highest similarity between these sequences lies in the C-terminal zinc-finger DNA binding domain (Figure 2B). This region contains the two CCHC motifs for coordinating the zinc atoms that are completely conserved in *Amnos*. Outside of this region, sequence similarity is limited to short sequences. Phylogenetic analysis of a multiple alignment of this conserved DNA binding domain (Figure 2C) clusters the predicted *Amnos* protein sequence with a predicted *nanos* gene from *Nasonia*, and *Drosophila nanos*. Other insect *nanos* sequences (*Schistocerca*, *Aedes* and *Anopheles*), form a separate clade with mouse *nanos* 2 and 3 and *Danio nanos*. Mouse *nanos*1 and human *nanos* 1 sequences cluster with the *Drosophila* sequence and cnidarian *nanos* sequences form a separate cluster.

In *Drosophila* the 3'UTR of *nanos* contains a structural RNA motif required for translational repression [39,41], no sequence homology to this region can be seen in the *Amnos* 3'UTR. An RNA structure prediction program (MFOLD[42]) predicts the first 130 bases of the *Amnos* 3'UTR produces a similar RNA fold to that of the *Drosophila* 3'UTR (Figure 2A ii and iii). The *Drosophila* motif contains sequences similar to bearded boxes that are also associated with translation repression [43]. Predicted

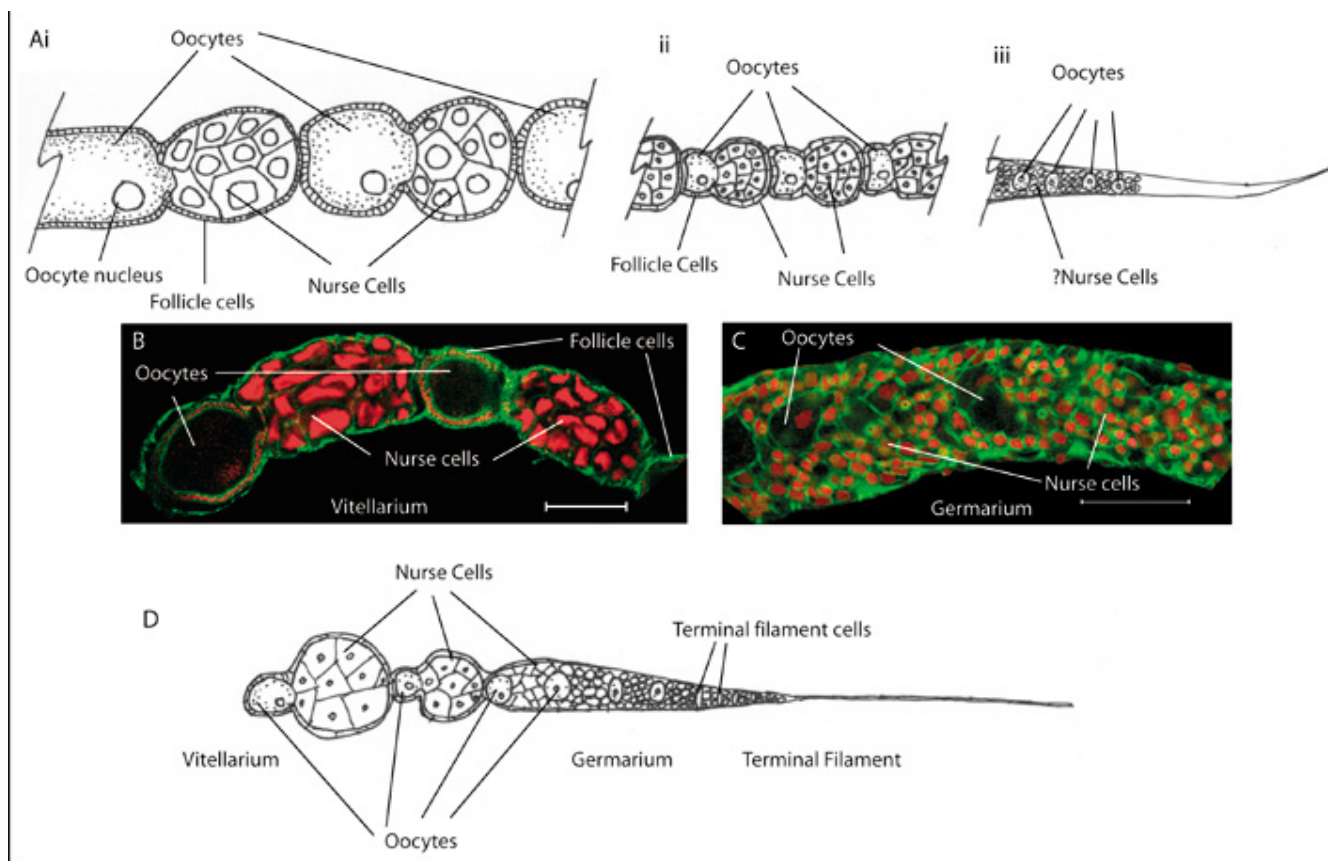


Figure 5
Structure of the Honeybee ovary. A) Diagram of the morphology of an ovariole from a mated queen bee ovary. i) the late vitellarium, ii) the early vitellarium, iii) the germarium and terminal filament (the placement of germ cells in the terminal filament is unclear and thus not diagrammed). B) A projection of 25 confocal Z sections through the late germarium of a mated honeybee queen ovariole stained for DNA using Propidium iodide (red) and cortical actin using Alexa fluor 488 phalloidin (green). C) A single confocal section through the germarium of a honeybee queen ovariole, stained as per B, Note the circular actin-rich structures, possibly ring canals. D) Diagram of the morphology of a worker bee ovariole.

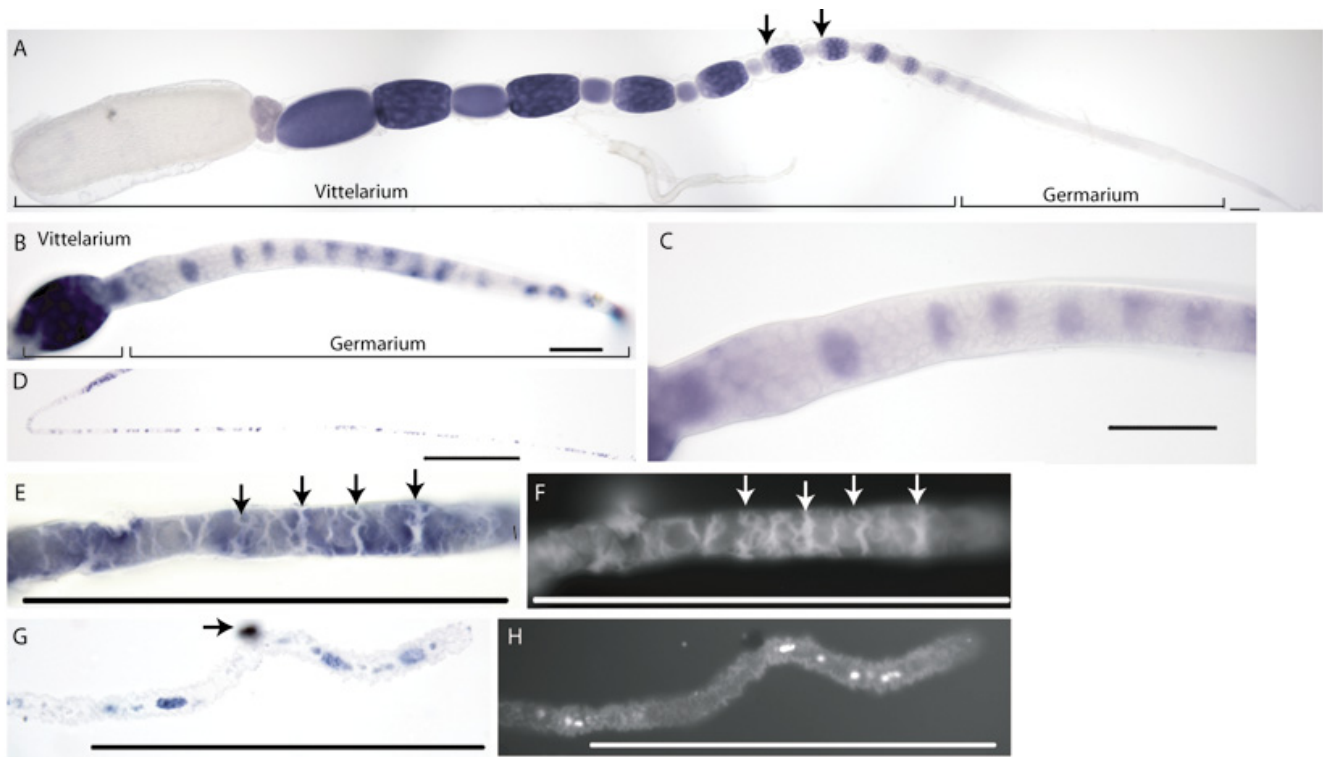
bearded boxes are present downstream of the *Amnos* gene but not in the predicted secondary structure.

Expression of *Amvasa* in honeybee embryos

In-situ hybridisation using a fragment of the *Amvasa* cDNA sequence was used to determine the placement and development of PGCs in female honeybee embryo (Figure 3). The probe was made from a 631 bp fragment of *Amvasa* from the second exon, a relatively non-conserved region of the gene. The C terminal part of the protein encoded by this fragment is boxed in Figure 1. *Amvasa* RNA is present at early stages of *Apis* embryogenesis, with a uniform distribution at stage 1 (Staging as per [35]) (Figure 3A and 3B). This initial expression rapidly disappears by late stage 1. *Amvasa* is not expressed in any cells until stage 9, when *Amvasa* RNA appears in a line of cells start-

ing in the 3rd abdominal segment (A3) and stretching to the 6th abdominal segment (A6) (Figure 3G and 3N). This line of cells lies just ventral to the boundary between the germband and the extra-embryonic membranes and lies just underneath the epidermis of the embryo. These cells continue to express *Amvasa* through the rest of embryogenesis (Figure 3I and 3K), and, in larval honeybees, come to be placed in regions consistent with the placement of the genital ridge (Figure 3L).

Amvasa RNA is also detected in a small group of cells in the mandibular segment in stage 9 embryos (Asterisks in figure 3G and 3I and arrow in Figure 3O). It is unclear what this structure is.

**Figure 6**

Expression of *Amvasa* in Honeybee ovaries. Scale bars represent 100 micrometers. A) The full length of a mated queen ovariole stained for *Amvasa* RNA. *Amvasa* RNA is present in both nurse cells and oocytes but not follicle cells. Staining is absent from the oldest oocytes, an artefact caused by problems getting labelled probes into these oocytes. All other oocytes have uniform staining of *Amvasa* RNA except those in the germarium and terminal filament where *Amvasa* RNA is absent. *Amvasa* RNA is also present in the nurse cells. In the early vitellarium, the nurse cells closest to the oocyte express lower levels of *Amvasa* RNA (arrows), but this difference in expression is not seen in the late vitellarium. *Amvasa* RNA levels are reduced in the last set of nurse cells, which are degrading prior to fertilisation and oviposition of the egg. B) *Amvasa* RNA expression in the vitellarium and germarium of a worker bee ovary. In the vitellarium, *Amvasa* RNA is expressed in the nurse cells and oocyte. In the germarium, *Amvasa* RNA is restricted to the oocytes themselves, except for the last oocyte/ nurse cell group, where *Amvasa* is expressed in some of the nurse cells. C) Magnification of the germarium shown in B under DIC optics showing *Amvasa* expression in germ-cells and in the last group of nurse cells (arrowed) D) The tip of the germarium and the terminal filament of a worker bee ovary stained for *Amvasa* RNA. *Amvasa* expression appears in a subset of cells in each tissue, presumably PGCs. E and F) Magnification of the beginning of the terminal filaments of a worker bee ovary stained for *Amvasa* RNA and using the nuclear dye DAPI under brightfield (E) and fluorescence (F) optics. *Amvasa* RNA is seen in a subset of cells in this region. The terminal filament cells (arrowed) have no *Amvasa* expression. G and H) a region of terminal filament from a worker bee ovary stained for *Amvasa*, and using the nuclear dye DAPI under brightfield (G) and fluorescence (H) optics. Cells expressing *Amvasa* can be seen surrounded by non-staining cells. *Amvasa* expression quenches the nuclear signal in these images, so bright spots of DAPI fluorescence indicate unstained nuclei.

The expression of *Amvasa* RNA seems to indicate that PGCs in *Apis* form late in embryogenesis. It is possible, however, that the distribution of the *Amvasa* RNA does not reflect the distribution of *Amvasa* protein, as in *Drosophila* [44]. To determine if *Amvasa* protein is present in embryo stages 2–8, embryos were stained using an antibody raised against *Schistocerca gregaria* vasa that has been shown to cross react in a number of species [7,13,25]. No staining above background in *Apis* embryos or ovaries was

detected using the antibody in conditions in which *Drosophila melanogaster* embryos did stain in the canonical vasa pattern, implying this antibody does not cross-react with *Amvasa* (data not shown).

To confirm the timing of *Amvasa* RNA expression, RT-PCR was carried out on RNA from honeybee embryos of various stages (Figure 3P). A PCR product of the target length, consistent with *Amvasa* RNA, was found only in RNA from



Figure 7
Expression of *Amnos* in honeybee ovaries. Scale bars represent 100 micrometers. A) Ovariole from a mated queen bee stained for *Amnos* RNA. *Amnos* RNA is present in both oocytes and a subset of nurse cells. In oocytes in the late vitellarium, *Amnos* RNA is present in a localised domain in the posterior regions of the oocyte, with a 'tail' of *Amnos* RNA spreading back along one side of the oocyte towards the anterior. In the early vitellarium, *Amnos* RNA is localised in a domain in central regions of each oocyte. *Amnos* is also expressed weakly in the nurse cells, particularly in those closest to the oocyte. B) DIC image of an oocyte and nurse cells from the early vitellarium showing the localised domain of *Amnos* and the nucleus (asterisk). The patch of *Amnos* RNA is not associated with the nucleus. C) DIC image of the basal germarium of an ovariole stained for *Amnos* RNA. In the germarium, oocytes contain *Amnos* RNA. In late germarium, this is in a patch as seen in the vitellarium. In earlier oocytes, expression appears uniform. The patch of *Amnos* RNA is not associated with the oocyte nucleus (asterisks). No expression of *Amnos* is seen in the nurse cells. D) DIC image of an oocyte and nurse cells from the late vitellarium. *Amnos* RNA is only present in a small posterior patch, not associated with the nucleus (asterisk). The nurse cells all express *Amnos* weakly. E and F) DIC images of two focal planes through an oocyte in the vitellarium stained for *Amnos* RNA. *Amnos* RNA is present in a streak running down the oocyte, with the highest and most defined expression in the posterior. The streak and domain is not associated with the oocyte nucleus (asterisk in E). G) Worker bee ovariole stained for *Amnos* RNA. Strong expression is seen in the nurse cells of the vitellarium, with weaker expression in the oocyte. In the germarium, *Amnos* RNA is weakly expressed in oocytes only. No expression is present in the terminal filament (data not shown).

just laid eggs and embryos over stage 10. No *Amvasa* RNA could be detected in embryos of stages 3–7, consistent with our in-situ hybridisation results.

Expression of *Amnos* in honeybee embryos

As *Amvasa* RNA expression indicated the late formation of PGCs and I was unable to confirm this using the anti-vasa

antibody, the expression of *Amnos* was used as a second PGC marker in female embryos. *Amnos* RNA is first detected in stage 1 embryos where it is present in a gradient with highest expression in posterior regions of the embryo (Figure 4A). This expression fades rapidly through embryogenesis such that it is faint in stage 2 embryos (Figure 4C) and absent from stage 3 embryos. No further expression of *Amnos* is seen in embryos until stage 9 (Figure 4I), when expression appears in a line of cells, starting in the 3rd abdominal segment and stretching to the 6th abdominal segment. Like the cells marked with *Amvasa*, *Amnos* expressing cells lie under the epidermis and just ventral to the boundary between the embryonic and extraembryonic membranes. The distribution of *Amnos* and *Amvasa* in these embryos is identical except for the anterior patch of cells expressing *Amvasa*; these cells do not express *Amnos*.

To confirm the timing of *Amnos* RNA expression, RT-PCR was carried out on RNA from honeybee embryos of various stages (Figure 4K). A PCR product of the target length (257 bp), consistent with *Amnos* RNA, was found only in RNA from just-laid eggs and embryos over stage 10. These data are consistent with our in-situ hybridisation results in that *Amnos* RNA is not expressed between stages 3–7.

Expression of *Amvasa* in queen and worker bee ovaries

Apis mellifera have polytrophic meroistic ovaries, similar to those of *Drosophila melanogaster*. Despite this similarity, a number of differences in morphology and biology exist. One particularly important difference is that worker bee ovaries have many less ovarioles than those of queens (Figure 5). Workers in a queenright colony have small ovaries that are chemically repressed by the presence of the queen and her eggs [45]. Removal of the queen from a colony can cause the reactivation of the worker bee ovaries and the workers may lay eggs [45]. To see if germ cell placement or organisation is significantly different in workers compared to queen ovaries I used in-situ hybridisation for *Amvasa* RNA to examine the placement of germ cells in both types of ovary (Figure 6).

In the queen ovary, both nurse and germ cells express *Amvasa*. *Amvasa* RNA is not localised to any particular area in either cell type. *Amvasa* RNA is present in oocytes in the vitellarium, but expression is absent, or very weak in the germarium and terminal filament. In the nurse cells, RNA is present in all cells in the vitellarium, but in the germarium, the expression of *Amvasa* RNA is reduced in the nurse cells closest to the oocytes (arrows in figure 6A). Expression of *Amvasa* RNA does not extend beyond the germarium in nurse cells. No expression of *Amvasa* is seen in follicle cells.

In worker ovaries a similar expression pattern can be seen (Figure 6B). *Amvasa* RNA is present in all oocytes, but, in contrast to queen ovaries, this expression extends into the terminal filament (Figures 6D, E, F, G, and 6H). Expression of *Amvasa* RNA in the nurse cells is reduced in worker ovaries. Expression can be seen in nurse cells in the vitellarium, and, faintly, in the nurse cells associated with the last oocyte in the germarium (Figure 6C). In the terminal filament (Figures 6D, E, F, G and 6H) *Amvasa* RNA expression marks germ cells in the entire structure. Comparison of the expression of *Amvasa* RNA and the location of nuclei stained with DAPI indicates that the germ cells in the start of the terminal filament are surrounded by disc shaped 'terminal filament cells' [46] that do not stain for *Amvasa*. However within these compartments, cells that do not express *Amvasa* are also present (Figure 6E and 6F). It is not clear if these are nurse cells or follicle cells. In the last sections of the terminal filament, cells positive for *Amvasa* RNA are interspersed with cells that are not (Figure 6G and 6H). The expression of *Amvasa* in the worker, but not queen terminal filament is a clear difference between castes.

Expression of *Amnos* in queen and worker bee ovaries

The expression of *Amnos* RNA in queen and worker bee ovaries was also examined using in-situ hybridisation (Figure 7). In the early vitellarium and germarium, *Amnos* RNA is present only in a discrete domain in the anterior region of each oocyte, anterior to the oocyte nucleus (Asterisks in figure 7C). In later vitellaria, *Amnos* RNA staining is found faintly in the nurse cells, and in the localised domain in each oocyte. In later vitellaria, however, this domain lies on one side of the oocyte and appears as a patch in posterior regions of the egg with a more diffuse 'tail' stretching back to the more posterior regions. Neither the patch nor the tail are associated with the oocyte nucleus (asterisks in Figure 7B, C, D and 7E) and in all cases lies posterior to it. In the oldest oocytes (Figure 7D) *Amnos* RNA is found in an anterior patch that is fainter than that in younger oocytes. I believe this faint staining is an artefact caused by differential permeability of old and young oocytes. The 'comet-like' form of the patch of localised *Amnos* RNA in later vitellaria is consistent with movement of a discrete focus of *Amnos* RNA from anterior of the oocyte nucleus to posterior regions of the oocyte.

In worker bee ovaries, by contrast, *Amnos* RNA is weakly and uniformly distributed in oocytes in the vitellarium and germarium (Figure 7G[47]) and, at higher levels, in the nurse cells of the oldest oocyte. *Amnos* RNA is not expressed in nurse cells, follicle cells or cells in the terminal filament.

Discussion

Identification of *Apis* homologues of *nos* and *vasa*

Blast searches and phylogenetic reconstruction indicate that I have identified honeybee homologues of the evolutionarily conserved germ-cell markers *nanos* and *vasa*.

The *Amvasa* gene, also identified by [10], has all of the conserved sequences indicative of a *vasa* protein, and clusters with other insect *vasa* sequences to the exclusion of non *vasa* DEAD box helicases. The sequence does, however, have significant variation in the EXRKF motif, that is conserved in PL10 and *vasa* helicases. The function of this motif is unknown, thus the significance of its absence from *Amvasa* is not known.

The *Amnos* gene identified in this study is similar in C-terminal regions to *nanos* proteins from other species. This region contains the zinc coordination residues of the zinc fingers. These residues are completely conserved in *Amnos*. *Drosophila nanos* contains a sequence that forms a specific secondary structure in the 3'UTR that is required for translational repression [41]. This secondary structure is bound by the *smaug* protein in early embryos [39]. A similar secondary structure is predicted to form in the *Amnos* 3'UTR though its function, if any, is unknown.

The conservation and phylogenetic placement of these sequences is consistent with them encoding the conserved germ-cell markers *vasa* and *nos*.

Germ cell development in *Apis mellifera*

Expression of *Amnos* and *Amvasa* RNA occurs in two phases of honeybee embryonic development. Both genes are expressed early in development, *Amvasa* in the entire embryo, and *Amnos* in a gradient pattern with highest concentrations in the posterior of the embryo, both probably due to maternal RNA contribution. These expression patterns disappear by stage 2. In later development, at and after stage 9, both genes are expressed in a subset of cells that lie close to the dorsal boundary of the embryo proper and the extraembryonic membranes. These cells eventually come to be located in the dorsal-most regions of the embryo consistent with the genital ridges and forming gonads. That both genes are not expressed in embryos between stage 2 and stage 9 implies that during these stages there are no PGCs in the honeybee embryo, and that these cells form via an inductive event at, or just before, stage 9.

Neither morphological studies, nor the use of molecular markers has found any evidence for pole-cells, pole plasm or early segregating PGCs [8]. This is unlike the formation of PGCs in *Drosophila*, which has a similar long-germ form of embryo to that of *Apis*.

The expression of these two PGC markers implies that germ-cells form very close to the boundary of the embryo proper and the extra-embryonic membranes. The cells form in A3-A6 and no sign of migration of the PGCs can be seen. As dorsal closure occurs, the cells appear to be moved, passively, to the dorsal surface of the larva, close to the dorsal midline.

These findings indicate two possible scenarios for the origin of PGCs in the honeybee embryo. It is possible that the appearance of PGC markers at a late stage of embryogenesis, similar to the situation in mice [48,49] implies that an inductive event at stage 9 specifies PGCs. The organisation of the cells, in a single line, is what might be expected if the cells are being induced by a signal released from an almost linear source. Just dorsal of the PGCs are the extra-embryonic membranes that cover the dorsal opening. It is possible that the putative inducing signal leading to the formation of PGC fate emanates from the extra-embryonic membranes.

It is also possible that the *Amvasa* and *Amnos* RNA are not expressed in germ-cells that do form early in development, and only come to be expressed when these cells reach the abdomen. *Drosophila vasa* RNA expression is indeed absent from PGCs until stage 12 [44] and *nanos* expression is down-regulated in *Drosophila* after stage 10 [38]. In the crustacean *Parhayle* PGCs are specified very early in development, without the expression of *vasa* [13]

This study is unable to determine which of these two possible mechanisms underlies germ-cell formation in Honeybees.

Localisation of *nos* RNA and posterior development in *Apis mellifera*

In *Drosophila* and other insects *nanos* acts to regulate posterior development [38]. *Nos* expression has been studied in both *Drosophila* [38,41], other dipterans [22,23] and *Schistocerca* [36]. In each studied species *nos* RNA is localised in posterior regions in early development. In *Drosophila* most *nos* RNA is localised very tightly to the posterior, and its translation is repressed in anterior regions [41]. In *Drosophila* *nos* acts as a key regulator of posterior development, translationally repressing, in concert with *pumilio*, the anterior gene *hunchback* in the posterior [37,50]. In *Schistocerca*, the expression of *nos* is coincident with a clearance of *hunchback* expression from the very posterior regions of the embryo [36]. *Hunchback* mRNA from *Schistocerca* is predicted to contain a *nanos* response element, similar to that which is bound by *Drosophila* *nanos* to repress *hunchback* translation.

In Honeybees *Amnos* RNA is present in the posterior of the early embryo (appearing in just laid eggs and clearing in

stage 2) This domain is broader than that in *Drosophila* where the highest levels of *nos* RNA form, and appears to represent a concentration gradient, with highest RNA expression in the very posterior of the embryo. The *Amnos* 3'UTR does not contain an element with sequence similarity to the stem-loop structure in the 3'UTR of *Drosophila nos*, but a similar RNA structure is predicted to be formed by it.

In the ovary of queen bees, *Amnos* RNA expression is found in a localised patch in oocytes, that appears to move, during oocyte maturation, from anterior to posterior. In *Schistocerca* [36], and mosquitoes [22] *nanos* RNA is also localised tightly to posterior regions of the oocyte. The localisation of *Amnos* RNA in the oocyte is consistent with a role in posterior patterning.

Conclusion

I have used *Amvasa* and *Amnos* as molecular markers of PGC fate in the honeybee *Apis mellifera*. The timing of placement of their expression provides no evidence for early specification of germ-cells but implies that honeybee PGCs form from an inductive event late in embryonic development. It is also possible that germ-cells form early in the Honeybee embryo but do so in the absence of *vasa* or *nanos* expression. Both these possibilities differ from PGC specification in *Drosophila*, where morphologically distinct PGCs appear early in development and are marked by the expression of *vasa* and *nanos*, or the more closely related *Copidosoma*, where a cellular organelle determines PGC fate and contains *vasa* protein [5].

Any putative induction process implied by these results appears similar to the segregation of PGCs in *Schistocerca*, where early segregation is also not seen. The placement of PGCs, forming close to the border between extra-embryonic and embryonic tissue and inside the epidermal layer, seems similar in both species.

This mode of PGC formation indicates that the evolutionary history of PGC segregation in insects is not simple, with different mechanisms acting in different species. It is unclear, at this point, what the ancestral mechanism is, and how the different mechanism of PGC specification in extant insects may have arisen.

Methods

Beekeeping

Apis mellifera were cultured using standard techniques in Dunedin, New Zealand. Honeybee embryos were collected from frames removed from nucleus boxes containing small honeybee colonies.

Gene identification and phylogenetics

Homologues of the *Drosophila melanogaster vasa* and *nanos* genes were identified in the Honeybee genome sequence (version 2) using tBlastN searches [40]. Regions of the genome with significant blast hits were extracted and blasted back to the *Drosophila melanogaster* genome. Gene predictions from either NCBI (using gnomon) or ENSEMBL were then examined in the regions with reciprocal top blast hits for *vasa* and *nanos*. Primers for amplification from cDNA were designed to the regions with the highest homology.

Multiple alignments of the predicted *Apis* genes with homologous genes from other species were carried out using ClustalX [51]. Phylogenetic analysis was performed on these multiple alignments using MrBayes 3.1 [52] or Phylip [47]

Molecular Cloning

Ovaries were dissected from mated queen bees in PBS and poly A+ RNA extracted using a quickprep RNA extraction kit (GE Biosciences). cDNA was generated from this RNA using superscript II reverse transcriptase (Invitrogen) and an oligo-DT primer following the manufacturers instructions. PCR was performed on this cDNA using primers for *vasa* (tggcaatgtaacgataaaaagacc, *Amvasa*RNA5' tgggcgacacgatgacaac, *Amvasa*RNA3') or *nanos* (gtcctcacacgcaccacaa, *AmNanos*5' acgccgaagaaaaataagaagaaac, *AmNanos*3'). PCR products of the correct size were cloned into pGEM-T-Easy (Promega) following the manufacturers instructions. Plasmid DNA was isolated from these clones and sequenced at the Allan Wilson Centre for Molecular Ecology and Evolution.

RNA for RT-PCR experiments was extracted with an RNAeasy kit (Qiagen), treated with RNase free DNase (Invitrogen) for 2 hours at 37 degrees, heated to 65 degrees for 30 minutes, and precipitated with ammonium acetate and isopropanol. First strand synthesis and PCR were performed as above with primers for *Amvasa* (gcgtttccaccatcatc and gttgtcatcgtgtgccca) and *Amnos* (as above).

In-situ hybridisation and antibody staining

In-situ hybridisation was performed on ovaries and embryos as described in Osborne and Dearden [53] and antibody staining after in-situ hybridisation using the 4D9 anti-engrailed-like antibody [54] as described in Osborne and Dearden [55].

Confocal microscopy

Ovaries were dissected from mated queen bees and fixed in a mixture of 4% formaldehyde in PBS: Heptane overnight. Ovaries were washed in methanol and rehydrated in PTw (PBS + 0.1% Tween 20). The ovaries were treated with 1 µg/ml RNase A and PTw for one hour, and then

treated with 0.5 µg/ml Propidium iodide and 0.33 mM Alexa-Fluor 488 Phalloidin (Molecular probes) overnight in PTw. The ovaries were washed in PTw four times over 30 minutes and individual ovarioles dissected from the ovary mass. Individual ovarioles were mounted on microscope slides in 70% glycerol. Confocal imaging was carried out using a Leica confocal microscope.

Authors' contributions

PKD conceived and carried out the experiments discussed above and drafted the manuscript.

Acknowledgements

I would like to thank Cassandra Extavour and Michael Akam for kind provision of their anti-*vasa* antibody, Sue Heath and Prof. Alison Mercer for their help with Honeybee rearing and Megan J Wilson, Andrew G Cridge and E P Dearden for their critical reading of this manuscript. I would also like to thank Dr Craig Marshall and the Chemistry and Botany Departments, University of Otago for providing space for Honeybee culture. The anti-engrailed monoclonal antibody developed by Corey Goodman was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. This work was supported by a University of Otago Research Grant and a Royal Society of New Zealand Marsden Fund Grant (UOO0401).

References

- Anderson: **Embryology and phylogeny in annelids and arthropods.** Pergamon Press; 1973.
- Warrior R: **Primordial germ cell migration and the assembly of the *Drosophila* embryonic gonad.** *Dev Biol* 1994, **166(1)**:180-194.
- Zissler D: **From egg to pole cells: ultrastructural aspects of early cleavage and germ cell determination in insects.** *Microsc Res Tech* 1992, **22(1)**:49-74.
- Klag J, Bilinski S: **Oosome formation in two ichneumonid wasps.** *Tissue and Cell* 1993, **25(1)**:121-128.
- Grbic M, Nagy LM, Strand MR: **Development of polyembryonic insects: a major departure from typical insect embryogenesis.** *Dev Genes Evol* 1998, **208(2)**:69-81.
- Hunter MS, Nur U, Werrren JH: **Origin of Males by Genome Loss in an Autoparasitoid Wasp.** *Heredity* 1993, **70**:162-171.
- Chang C, Dearden P, Akam M: **Germ line development in the grasshopper *Schistocerca gregaria*.** *Developmental Biology* 2002, **252(1)**:100-118.
- Nelson JA: **The embryology of the Honeybee.** Princeton, Princeton University Press; 1915.
- Nakao H: **Isolation and characterization of a *Bombyx* *vasa*-like gene.** *Development Genes and Evolution* 1999, **209(5)**:312-316.
- Donnell DM, Corley LS, Chen G, Strand MR: **Caste determination in a polyembryonic wasp involves inheritance of germ cells.** *Proc Natl Acad Sci U S A* 2004, **101(27)**:10095-10100.
- Dearden PK, Grbic M, Donly C: ***Vasa* expression and Germ Cell Specification in the Spider mite *Tetranychus urticae*.** *Development, Genes and Evolution* 2003, **212(12)**:599-603.
- Sagawa K, Yamagata H, Shiga Y: **Exploring embryonic germ line development in the water flea, *Daphnia magna*, by zinc-finger-containing *VASA* as a marker.** *Gene Expr Patterns* 2005, **5(5)**:669-678.
- Extavour CG: **The fate of isolated blastomeres with respect to germ cell formation in the amphipod crustacean *Parhyale hawaiiensis*.** *Dev Biol* 2005, **277(2)**:387-402.
- Roussel DL, Bennett KL: ***glh-1*, a germ-line putative RNA helicase from *Caenorhabditis*, has four zinc fingers.** *Proc Natl Acad Sci U S A* 1993, **90(20)**:9300-9304.
- Lasko PF, Ashburner M: **The product of the *Drosophila* gene *vasa* is very similar to eukaryotic initiation factor-4A.** *Nature* 1988, **335(6191)**:611-617.
- Castrillon DH, Quade BJ, Wang TY, Quigley C, Crum CP: **The human *VASA* gene is specifically expressed in the germ cell lineage.** *Proc Natl Acad Sci U S A* 2000, **97(17)**:9585-9590.
- Yoon C, Kawakami K, Hopkins N: **Zebrafish *vasa* homologue RNA is localized to the cleavage planes of 2- and 4-cell-stage embryos and is expressed in the primordial germ cells.** *Development* 1997, **124(16)**:3157-3165.
- Tanaka SS, Toyooka Y, Akasu R, Katoh-Fukui Y, Nakahara Y, Suzuki R, Yokoyama M, Noce T: **The mouse homolog of *Drosophila Vasa* is required for the development of male germ cells.** *Genes Dev* 2000, **14(7)**:841-853.
- Mahowald AP: **Assembly of the *Drosophila* germ plasm.** *Int Rev Cytol* 2001, **203**:187-213.
- Styhler S, Nakamura A, Swan A, Suter B, Lasko P: ***vasa* is required for GURKEN accumulation in the oocyte, and is involved in oocyte differentiation and germline cyst development.** 1998, **125**:1569--1578.
- Kobayashi S, Yamada M, Asaoka M, Kitamura T: **Essential role of the posterior morphogen *nanos* for germline development in *Drosophila*.** 1996, **380**:708--711.
- Calvo E, Walter M, Adelman ZN, Jimenez A, Onal S, Marinotti C, James AA: ***Nanos (nos)* genes of the vector mosquitoes, *Anopheles gambiae*, *Anopheles stephensi* and *Aedes aegypti*.** *Insect Biochemistry and Molecular Biology* 2005, **35(7)**:789-798.
- Curtis D, Apfeld J, Lehmann R: ***nanos* is an evolutionarily conserved organizer of anterior-posterior polarity.** *Development* 1995, **121(6)**:1899-1910.
- Schaner CE, Deshpande G, Schedl PD, Kelly WG: **A conserved chromatin architecture marks and maintains the restricted germ cell lineage in worms and flies.** *Dev Cell* 2003, **5(5)**:747-757.
- Extavour CG, Pang K, Matus DQ, Martindale MQ: ***vasa* and *nanos* expression patterns in a sea anemone and the evolution of bilaterian germ cell specification mechanisms.** *Evol Dev* 2005, **7(3)**:201-215.
- Torrás R, Yanze N, Schmid V, Gonzalez-Crespo S: ***nanos* expression at the embryonic posterior pole and the medusa phase in the hydrozoan *Podocoryne carnea*.** *Evolution & Development* 2004, **6(5)**:362-371.
- Kang D, Pilon M, Weisblat DA: **Maternal and Zygotic Expression of a *nanos*-Class Gene in the Leech *Helobdella robusta*: Primordial Germ Cells Arise from Segmental Mesoderm.** *Dev Biol* 2002, **245(1)**:28-41.
- Agee SJ, Weisblat DA: **Investigating the embryological function of maternal *Hro-nos*, a *nanos* homolog in the leech *Helobdella robusta*.** *American Zoologist* 2001, **41(6)**:1377-1378.
- Tsuda M, Sasaoka Y, Kiso M, Abe K, Haraguchi S, Kobayashi S, Saga Y: **Conserved role of *nanos* proteins in germ cell development.** *Science* 2003, **301(5637)**:1239-1241.
- Jaruzelska J, Kotecki M, Kusz K, Spik A, Firpo M, Pera RAR: **Conservation of a *Pumilio-Nanos* complex from *Drosophila* germ plasm to human germ cells.** *Development Genes and Evolution* 2003, **213(3)**:120-126.
- Wang Z, Lin HF: ***Nanos* maintains germline stem cell self-renewal by preventing differentiation.** *Science* 2004, **303(5666)**:2016-2019.
- Hayashi Y, Hayashi M, Kobayashi S: ***Nanos* suppresses somatic cell fate in *Drosophila* germ line.** *Proceedings of the National Academy of Sciences of the United States of America* 2004, **101(28)**:10338-10342.
- Koprunner M, Thisse C, Thisse B, Raz E: **A zebrafish *nanos*-related gene is essential for the development of primordial germ cells.** *Genes & Development* 2001, **15(21)**:2877-2885.
- Zhurrov V, Terzin T, Grbic M: **Early blastomere determines embryo proliferation and caste fate in a polyembryonic wasp.** *Nature* 2004, **432(7018)**:764-769.
- DuPraw EJ: **The Honeybee Embryo.** In *Methods in Developmental Biology* Edited by: Wilt FH, Wessells NK. New York, Thomas Y Cromwell Company; 1967:183-217.
- Lall S, Ludwig MZ, Patel NH: ***Nanos* Plays a Conserved Role in Axial Patterning outside of the Diptera.** *Curr Biol* 2003, **13(3)**:224-229.
- Irish VF, Lehmann R, Akam M: **The *Drosophila* posterior-group gene *nanos* functions by repressing hunchback activity.** 1989, **338**:646--648 %O BIOSIS ID: 36120474 %O PubMed: 2704419.

38. Wang C, Lehmann R: **Nanos is the localized posterior determinant in Drosophila.** *Cell* 1991, **66(4)**:637-647.
39. Dahanukar A, Walker JA, Wharton RP: **Smaug, a novel RNA-binding protein that operates a translational switch in Drosophila.** 1999, **4**:209-218.
40. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ: **Basic local alignment search tool.** *Journal of Molecular Biology* 1990, **215(3)**:403-410.
41. Dahanukar A, Wharton RP: **The Nanos gradient in Drosophila embryos is generated by translational regulation.** 1996, **10**:2610-2620.
42. Zuker M, Mathews DH, Turner DH: **Algorithms and thermodynamics for RNA secondary structure prediction.** In *A Practical guide in RNA biochemistry and biotechnology* Edited by: Barciszewski J, Clark BFC. Kluwer Academic Publishers; 1999.
43. Duchow HK, Brechbiel JL, Chatterjee S, Gavis ER: **The nanos translational control element represses translation in somatic cells by a Bearded box-like motif.** *Developmental Biology* 2005, **282(1)**:207-217.
44. Hay B, Jan LY, Jan YN: **A protein component of Drosophila polar granules is encoded by vasa and has extensive sequence similarity to ATP-dependent helicases.** *Cell* 1988, **55(4)**:577-587.
45. Katzav-Gozansky T, Soroker V, Ionescu A, Robinson GE, Hefetz A: **Task-related chemical analysis of labial gland volatile secretion in worker honeybees (Apis mellifera ligustica).** *J Chem Ecol* 2001, **27(5)**:919-926.
46. Tanaka ED, Hartfelder K: **The initial stages of oogenesis and their relation to differential fertility in the honey bee (Apis mellifera) castes.** *Arthropod Structure & Development* 2004, **33(4)**:431-442.
47. Felsenstein J: **PHYLIP (Phylogeny Inference Package) version 3.6.** 2004.
48. Lawson KA, Dunn NR, Roelen BA, Zeinstra LM, Davis AM, Wright CV, Korving JP, Hogan BL: **Bmp4 is required for the generation of primordial germ cells in the mouse embryo.** *Genes Dev* 1999, **13(4)**:424-436.
49. Lawson KA, Hage WJ: **Clonal analysis of the origin of primordial germ cells in the mouse.** *Ciba Found Symp* 1994, **182**:68-84; discussion 84-91.
50. Sonoda J, Wharton RP: **Recruitment of Nanos to hunchback mRNA by Pumilio.** *Genes Dev* 1999, **13(20)**:2704-2712.
51. Thompson JD, Higgins DG, Gibson TJ: **CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice.** *Nucleic Acids Research* 1994, **22**:4673-4680.
52. Ronquist F, Huelsenbeck JP: **MrBayes 3: Bayesian phylogenetic inference under mixed models.** *Bioinformatics* 2003, **19(12)**:1572-1574.
53. Osborne P, Dearden PK: **Non-radioactive in situ hybridisation to honeybees embryos and ovaries.** *Apidologie* 2005, **36**:113-118.
54. Patel NH, Martín-Blanco E, Coleman KG, Poole SJ, Ellis MC, Kornberg TB, Goodman CS: **Expression of engrailed Proteins in Arthropods, Annelids, and Chordates.** *Cell* 1989, **58(September 8)**:955-968.
55. Osborne P, Dearden PK: **Expression of Pax group III genes in the Honeybee (Apis mellifera).** *Development, Genes and Evolution* 2005, **215**:499-508.

Publish with **BioMed Central** and every scientist can read your work free of charge

"BioMed Central will be the most significant development for disseminating the results of biomedical research in our lifetime."

Sir Paul Nurse, Cancer Research UK

Your research papers will be:

- available free of charge to the entire biomedical community
- peer reviewed and published immediately upon acceptance
- cited in PubMed and archived on PubMed Central
- yours — you keep the copyright

Submit your manuscript here:
http://www.biomedcentral.com/info/publishing_adv.asp

