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ORIGINAL PAPER

L. M. Kuervers · C. L. Jones · N. J. O'Neil
D. L. Baillie

# The sterol modifying enzyme LET-767 is essential for growth, reproduction and development in *Caenorhabditis elegans*

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**Abstract** The *let-767* gene encodes a protein that is similar to mammalian steroid enzymes that are responsible for the reduction of 17-beta hydroxysteroid hormones. *Caenorhabditis elegans* is incapable of the de novo synthesis of cholesterol. Therefore, this free-living nematode must extract cholesterol from its environment and modify it to form steroid hormones that are necessary for its survival. *C. elegans* is unable to survive in the absence of supplemental cholesterol, and is therefore sensitive to cholesterol limitation. We show that a mutation in *let-767* results in hypersensitivity to cholesterol limitation, supporting the hypothesis that LET-767 acts on a sterol derivative. Furthermore, *let-767* mutants exhibit defects in embryogenesis, female reproduction and molting. Although ecdysone is the major molting hormone in insects, there is as yet no evidence for ecdysone synthesis in *C. elegans*, suggesting that a different hormone is required for molting in *C. elegans*. Our results suggest that LET-767 modifies a sterol hormone that is required both for embryogenesis and for later stages of development.

**Keywords** Cholesterol · GATA-binding factor · Hormones · Molting · Reproduction

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L. M. Kuervers · C. L. Jones · N. J. O'Neil · D. L. Baillie (✉)
Department of Molecular Biology and Biochemistry,
Simon Fraser University, Burnaby,
British Columbia, V5A 1S6, Canada
E-mail: dbaillie@gene.mbb.sfu.ca
Fax: +1-604-2686590

*Present address*: L. M. Kuervers
Department of Developmental Biology, Stanford University,
279 Campus Drive, Stanford, CA 94305, USA

*Present address*: N. J. O'Neil
Sanger Centre, Wellcome Trust Genome Campus, Hinxton,
Cambridge, CB10 1SA, UK

## Introduction

Cholesterol is the basic building block of steroid hormones, which are required to regulate growth, development, and fertility (Bloch 1983). Steroidogenic enzymes modify hormones, which may undergo further modification before being used as a ligand by nuclear hormone receptors in a signaling pathway. Upon activation by a specific hormone, these receptors translocate to the nucleus, and regulate the expression of downstream target genes (reviewed in Yamamoto 1985). *Caenorhabditis elegans* contains more than 250 nuclear hormone receptors (Sluder et al. 1999). However, only a few of these receptors have been characterized, and little information is available on the enzymes that are involved in steroid hormone synthesis in this organism. As a result, not a lot is known about the hormonal regulation of *C. elegans* development.

*C. elegans* undergoes a cyclic molting process between each stage of development (Singh and Sulston 1978). A new cuticle is secreted by the hypodermis beneath the old cuticle before the old cuticle is shed. It is known that ecdysone is the major molting hormone in Drosophila (reviewed in Kozlova and Thummel 2000), but little is known about the genetic regulation of molting in *C. elegans*. However, *C. elegans* is unable to propagate under laboratory conditions without the supplemental addition of cholesterol (Brenner 1974). Animals grown without supplemental cholesterol exhibit a variety of developmental defects including delayed growth, reduced brood size, and germline and somatic gonad defects (Merris et al. 2003). An earlier report also indicated that a dumpy phenotype and molting defects (Yochem et al. 1999) are associated with cholesterol deficiency, although cholesterol may have not been completely eliminated from the media used in this study. The requirement for cholesterol for growth, development and reproduction, and possibly molting, suggests that, as in Drosophila, steroid hormones are required for these processes in *C. elegans*.

In this study, we show that the LET-767 protein is required for embryogenesis, reproduction, and molting during *C. elegans* development. Our results suggest that LET-767 is involved in the production of a hormone that is required for these processes.

## Materials and methods

### Nematode culture and growth conditions

All *Caenorhabditis elegans* strains used in this study were grown at 20°C using Nematode Growth Medium. The *Escherichia coli* strain OP50 was used as a source of food. Standard genetic manipulations and handling of strains were performed as described by Brenner (1974).

### *C. elegans* strains

The strains used in this study were either created in our laboratory or were obtained from the *Caenorhabditis elegans* Genetics Center (University of Missouri, Columbia, Mo.). These included: the wild-type N2 var. Bristol strain, BC4634 *sDp3(III;f); dpy-17(e164) sDf125(s2424) unc-32(e189)III*, BC4849 *sDp3(III;f); dpy-17(e164) let-767(s2819)ncl-1(e1865) unc-32(e189)III*, BC4981 *sDp3(III;f); dpy-17(e164) let-767(s2176) unc-32(e189)III*, BC4174 *sDp3(III;f); dpy-17(e164) let767(s2464) unc-32(e189)III*, BC4121 *sDp3(III;f); dpy-17(e164) unc-32(e189)/ dpy-17(e164) lin-12(n941) III, CB164 dpy-17(e164)III*, and CB1489 *him-8(e1489)IV*.

Each BC lethal or deficiency strain is lethal upon loss of the free duplication that covers the region containing *dpy-17*, *let-767* , and *ncl-1*. Therefore, loss of *sDp3* in these strains results in an arrested Dpy-Unc animal. Unc animals were picked from BC4121 for use as a control in the cholesterol deprivation experiment (see below). These animals are *dpy-17(e164) unc-32(e189); sDp3(III;f)*.

### Complementation tests

Complementation tests among the four mutant strains that are the focus of this study were performed as follows. *dpy-17 let-767 unc-32; sDp3* hermaphrodites were mated to wild-type N2 males. The resulting heterozygous *dpy-17 let-767$^{X}$ unc-32/ + + +; sDp3(+/-)* WT males were then mated to *dpy-17 let-767 $^{Y}$ unc-32/ dpy-17 let-767 $^{Y}$ unc-32; sDp3* hermaphrodites (where *X* and *Y* denote different alleles). The resulting *dpy-17 let-767 $^{X}$ unc-32/dpy-17 let-767 $^{Y}$ unc-32* Dpy-Unc progeny from the outcross were then examined for arrest stages.

### Construction of transgenic animals and rescue of transformants

Transgenic animals were constructed and transformation rescue was performed as described by Mello et al. (1991) with modifications adopted by Janke et al. (1997).

### Cloning of the *C. elegans let-767* gene

The cosmids C56G2 (Genbank Accession No. U23177) and C31H11 (Accession No. AC006613) were kindly provided as bacterial stocks by Dr. Alan Coulson (Medical Research Council, Hinxton, UK). The subclone pLK5, which contains C56G2.6, was isolated from C31H11 by digestion with *Eco* RV and *Hin* dIII (Gibco/BRL).

### Sequencing of the *let-767 (s2176)* allele

DNA was extracted from homozygous *dpy-17 let-767 (s2176) unc-32* hermaphrodites as described by Barstead et al. (1991) with modifications adapted from Williams et al. (1992). A 1467-bp fragment containing the entire *let-767* coding region was amplified by PCR and extracted from an agarose gel. Approximately 50 ng of DNA was sequenced using an ABI PRISM BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) with 3.2 pmol of each of four primers, resulting in four sequencing reactions containing sequence from both strands of the genomic DNA.

### Cloning *gfp* into the *let-767* gene

The *gfp* -containing construct pPD117.01 was kindly provided by Andrew Fire (Carnegie Institute of Washington, Washington D.C.). The region between the two GFP primers encodes the last 15 amino acids of the upstream gene C56G2.7, the promoter region of *let-767*, and all but the last 13 amino acids of C56G2.6 (LET-767). The upstream primer 767-GFP-F (5′-GCGGTGCAGG-CGCAACCGTCCAGACAACGAAGAT-3′) contains a *Pst* I site, and the downstream primer 767-GFP-R (5′-GCCGGTACCTT-GGCTTCTCTTTCCTTCTTTCTGA-3′) contains a *Kpn* I site, which allow for in-frame fusion with the *gfp* gene.

### Fluorescence microscopy

Ten to fifteen transgenic animals containing the *let-767::gfp* fusion construct were placed in 10 $\mu$l of M9 buffer containing 10 mM sodium azide (Fisher) on a standard microscope slide, and covered with a coverslip. GFP fluorescence was observed using a Zeiss LSM 410 Inverted Laser Scan Microscope and standard FITC filters.

### Culture in the presence of different cholesterol concentrations

Nematode Growth Medium was prepared as described by Lewis and Fleming (1995) with final concentrations of cholesterol of 0 mg/l, 2.5 mg/l, 5 mg/l, or 20 mg/l. L4 *dpy-17 let-767 unc-32; sDp3* Unc hermaphrodites were placed on plates containing either 0 mg/l, 2.5 mg/l, 5 mg/l, or 20 mg/l cholesterol. *dpy-17 let-767 unc-32* Dpy-Unc progeny derived from these worms were transferred onto plates containing the same concentration of cholesterol as those on which their mothers had been grown, in order to facilitate phenotypic analysis. Arrest stages of the Dpy-Unc progeny were scored one week later.

## Results

### *let-767* is maternally provided and required for development

Three alleles of *let-767* were isolated in two separate screens for lethal mutations located on chromosome III (Stewart et al. 1998; Vatcher 2000). These alleles were designated *s2176, s2819* and *s2464*. *s2819* was described as a mid-larval lethal, whereas *s2464* was described as a late-larval lethal (Stewart et al. 1998). *s2176*, which was previously uncharacterized, arrests at an early larval stage. Upon further investigation, we determined that the *s2464* allele is a maternal-effect lethal (mel). We will refer to *s2176*, *s2819* , and *s2464* as *let-767(early)*, *let-767(mid)* , and *let-767(mel)*, respectively. Complementation tests were performed among the three *let-767*

alleles and a deficiency that deletes the region containing *let-767*, *sDf125*. The results are shown in Table 1. The null phenotype of *let-767* appears to be early larval arrest, as indicated by the early larval arrest stage that is observed when any one of the three alleles is placed over the deficiency. An early larval arrest is in agreement with the phenotype obtained in RNA interference experiments that were previously performed using *let-767* genomic DNA (Gonczy et al. 2000). However since *let-767* is required during embryogenesis (see below), it is possible that the *let-767* null phenotype is embryonic lethality.

## Cloning of *let-767*

*let-767* was previously positioned in a small region of the physical map that is covered by eight overlapping cosmids (Stewart et al. 1998). One of these cosmids, C31H11, rescued the lethal phenotype of *let-767* (Janke et al. 1997). Subsequently, we localized *let-767* within the region of overlap between the cosmids C31H11 and C56G2. This overlap contains four predicted genes, including C56G2.6, which shows similarity to genes for the mammalian enzyme 17$\beta$-hydroxysteroid dehydrogenase type 3 (17$\beta$-HSD3). Using a plasmid containing the full-length C56G2.6, we were able to rescue each of the three *let-767* mutations. A plasmid containing the *let-767::gfp* fusion was also able to rescue *let-767*; therefore, the fusion protein can substitute for LET-767. We were able to confirm that *let-767* is C56G2.6 by sequencing the early larval lethal allele, *s2176*. This allele has a missense mutation which results in the alteration of a highly conserved glycine residue to an arginine, at position 152 in the predicted amino acid sequence.

## LET-767 is a steroid dehydrogenase

BLAST searches performed with the C56G2.6 protein sequence revealed significant matches with short-chain alcohol dehydrogenases from other organisms. The best match with a protein of known function is with the human enzyme 17$\beta$-HSD3 (Genbank Accession No. AAC50066), with 35% identity and 54% similarity. However, the most significant match with human proteins, 41% identity and 60% similarity, is with a related protein of unknown function. The alignment in Fig. 1 compares LET-767 with the most similar human protein, the most similar Drosophila protein, and the canonical human 17$\beta$-HSD3 protein. The Drosophila protein was found by performing a BLAST search on the Drosophila genome sequence at www.flybase.org. It is 39% identical and 58% similar to LET-767 and is also of unknown function.

17$\beta$-HSD3 is an enzyme that uses NADP(H) as a cofactor for the conversion of androstenedione to testosterone in the testis (Geissler et al. 1994). The NADPH binding site and the motif characteristic of short-chain dehydrogenases are shown in Fig. 1. These two motifs are highly conserved. Although no specific function has been attributed to the stretch of amino acids shown by the dotted underline in Fig. 1, it also is highly conserved and contains the glycine residue that is mutated in the *s2176* mutant (indicated by the asterisk in Fig. 1).

## *let-767* is a member of a family of steroid dehydrogenases in *C. elegans*

A BLAST search with the LET-767 protein sequence also revealed four highly similar genes for 17$\beta$-HSD3 enzymes in *C. elegans* (C06B3.4, C06B3.5, F11A5.12, and F25G6.5) (Fig. 1); all are located on chromosome V. The greatest similarity among all five proteins including LET-767 is between F11A5.12 and C06B3.4, which share 87% identity and 93% similarity along their entire length. C06B3.4 and F25G6.5 are the least similar, showing 63% identity and 79% similarity.

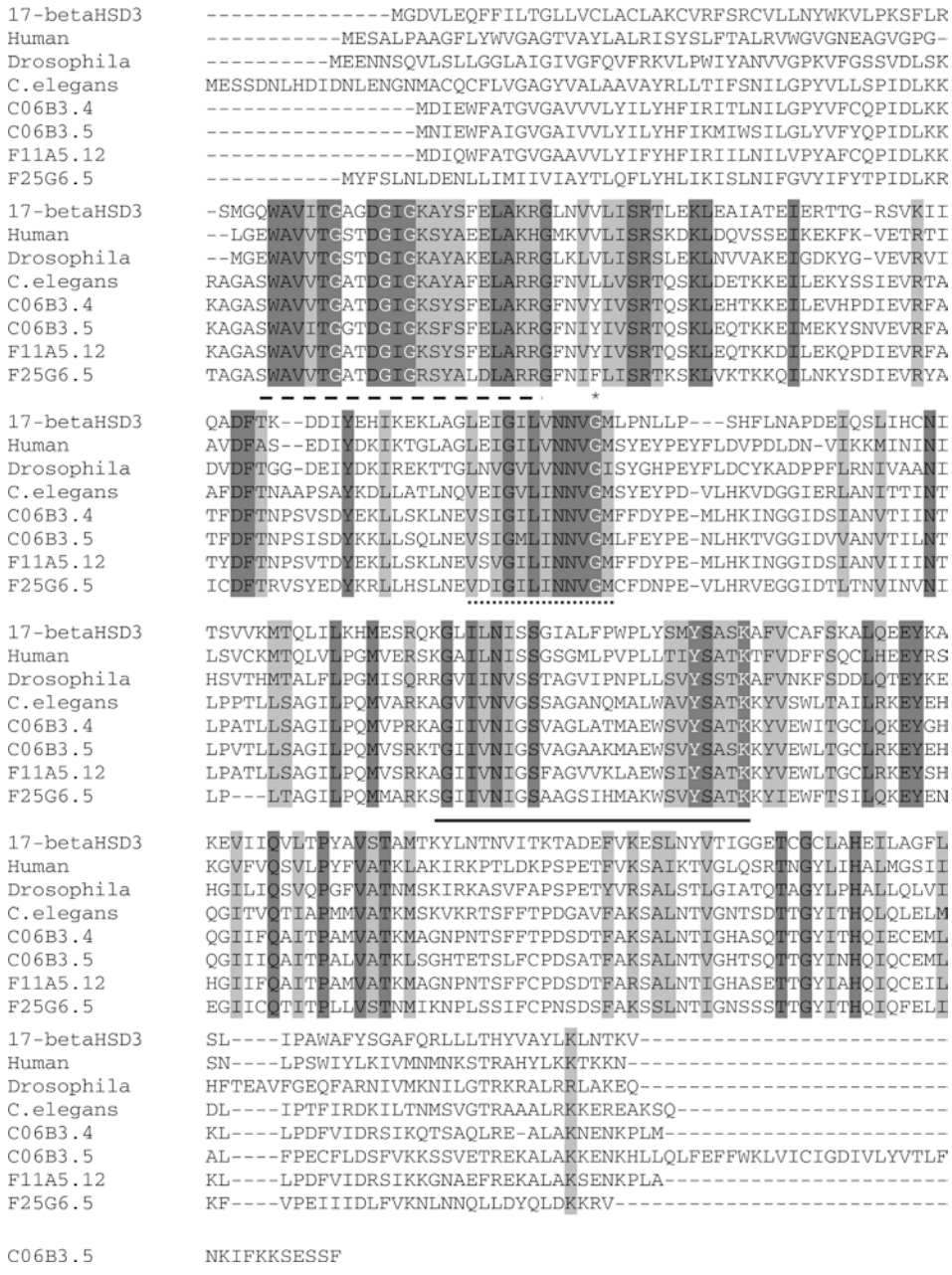
## *let-767::gfp* is detected in the gut

In order to observe the expression pattern of *let-767* we placed *gfp* under the control of the *let-767* promoter by fusing *gfp* to the *let-767* gene. Transgenic animals carrying this construct express *gfp* in the gut, with a notable absence of expression in the gut nuclei (Fig. 2). This pattern of expression is seen from the first larval stage through to adulthood in both hermaphrodites and males (data not shown). In agreement with the pattern of GFP expression, the *let-767* transcript is present from embryos to adults as revealed by RT-PCR using RNA preparations from successive developmental stages and

**Table 1** Complementation tests among the three *let-767* alleles and the deficiency *sDf125*

| Hermaphrodite[a] | Male[a] | | | |
|---|---|---|---|---|
| | s2176 (Early) | s2819 (Mid) | s2464 (Mel) | sDf125 (Embryonic) |
| s2176 | ND | ND | Early (12) | Early (26) |
| s2819 | ND | ND | ND | Early (41) |
| s2464 | Mel (33) | Mel (31) | ND | Early (17) |
| sDf125 | Early ( > 50) | Early ( > 50) | Early ( > 50) | ND |

[a]The arrest stages of homozygotes derived from the crosses indicated are listed. Numbers in*parentheses* indicate the numbers of animals scored. ND, the genetic cross was not performed




*let-767*-specific primers (data not shown). We did not detect GFP at any stage of embryogenesis (Fig. 2C). As in the case of *ges-1*, expression of which is also gut-specific (Edgar and McGhee 1986), two GATA sites are present in the *let-767* promoter, at positions –293 and +24 relative to the start codon. Studies on the *ges-1* promoter have shown that a GATA site is necessary for its gut-specific expression pattern (Fukushige et al. 1996).

## *let-767* mutant phenotypes are enhanced by cholesterol deprivation

We chose to observe the effects of cholesterol limitation on *let-767* mutants because LET-767 is similar to steroid processing enzymes. Wild-type animals are able to survive for at least a single generation without the supplemental cholesterol (Yochem et al. 1999; Merris et al. 2003). *let-767* homozygous mutant animals were raised on plates containing either 5 mg/l (amount added to Standard Growth Medium), 2.5 mg/l, or no added cholesterol, in order to observe any changes in the lethal phase. The resulting developmental arrest stages are shown in Fig. 3. The maternal-effect lethal allele of *let-767* was phenotypically enhanced by Reduction of the amount of cholesterol available in the medium enhanced the phenotype associated with the *let-767(mel)* allele. *let-767(mel)* hermaphrodites arrested before reaching adulthood when cholesterol was added at 2.5 mg/l, but arrested at an early larval stage when no cholesterol was added to the medium (Fig. 3C). The addition of excess

**Fig. 1** Sequence alignment of the LET-767 protein with 17 -HSD3 proteins, constructed with Clustal. Identical amino acids are shaded in *dark grey*, while similar amino acids are shaded in *light grey*. The conserved NADPH binding motif (GXXXGXG) that is found in all members of the 17 $\beta$-HSD3 family (Tsai-Morris et al. 1999) is indicated by the *dashed underline*; the conserved glycines in indicated in *white*. The *dotted underline* denots a highly conserved motif to which no function has yet been assigned. The glycine residue that is mutated in mutants bearing the *s2176* allele is shown in *white* and marked with an *asterisk*. The *continuous underline* marks the short-chain alcohol dehydrogenase signature motif as predicted by Prosite (PS00061). The YXXXK motif shown in *white* is found in all members of the short-chain alcohol dehydrogenase family (Tsai-Morris et al. 1999). The alignment includes two sequences from human: 17$\beta$-HSD3 represents the human 17$\beta$-HSD3 enzyme, while the second (Human) is the closest LET-767/ human match (GenBank Accession No. AAD44482). The Drosophila sequence represents the closest LET-767 homolog in *Drosophila melanogaster* (GenBank Accession No. AE003442). The remaining four proteins are encoded in the *C. elegans* genome and are designated as in GenBank (C06B3.4, Accession No. CAB01114; C06B3.5, CAB01115; F11A5.12, CAB07363; F25G6.5, AAC25798)

cholesterol (20 mg/l) had no effect on any of the *let-767* mutants (data not shown).

The mutant phenotype of *let-767(mid)* animals appeared to be attenuated when the cholesterol level in the medium was reduced, and they arrested later than they did under standard growth conditions. Although this result appears paradoxical, the number of animals chosen for this particular experiment was low. Furthermore, it is possible that homozygous mutant animals were damaged during the transfer causing an earlier arrest in the control experiment. However, it is also possible that this mutant responds positively to cholesterol limitation. One possible explanation is that the *let-767(mid)* allele either has a regulatory mutation that results in an increase in the level of LET-767 protein, or a mutation that results in more efficient substrate binding, or conversion of substrate to product. An excess of LET-767 activity could disrupt sterol homeostasis, resulting in growth disturbances. A reduction in the concentration of substrate available might alleviate such a perturbance of homeostasis, and thus improve survival.

## *let-767* mutants exhibit molting defects

In order to further investigate the role of *let-767* in development, we analyzed the phenotypes of the three alleles. Molting defects are seen in all three *let-767* homozygotes (Fig. 4). Each allele is associated with constriction and bunching of old cuticle in the head region (Fig. 4A, C and E), a phenotype which occurs at a frequency of 30–40% (more than 60 animals were observed for each allele). A more severe phenotype that is observed in approximately 10% of *let-767(mel)* homozygotes ($n > 20$) and 40% of *let-767(mid)* ($n > 50$) is the failure to detach the old cuticle from the body (Fig. 4D, F). Finally, over 60% of *let-767(mid)* homozygotes and 10–20% of *let-767(mel)* ($n > 100$) and *let-767(early)* ($n > 100$) homozygotes exhibit a pinching of the cuticle near the tail region (Fig. 4B).

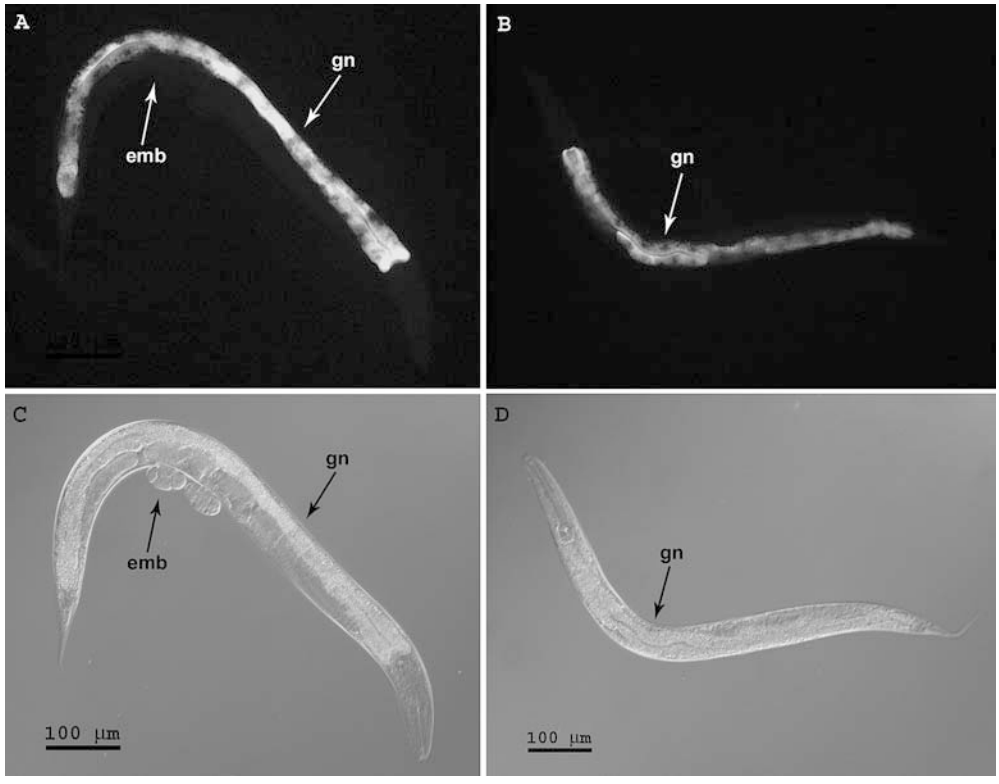



**Fig. 2A–D** Transgenic animals containing the *let-767::gfp* fusion construct. **A, B** GFP fluorescence. **C, D** Bright-field optics under visible light. Panel **C** was also illuminated to excite GFP fluorescence. GFP is present in the gut of the transgenic animals, but not in the embryos derived from transgenic adults. **A, C** Fertile adult transgenic hermaphrodite. **B, D** Transgenic larva. Multiple gut nuclei (gn) are visible in **A** and **B** as dark circles without gfp expression on either side of the gut lumen. emb, embryos

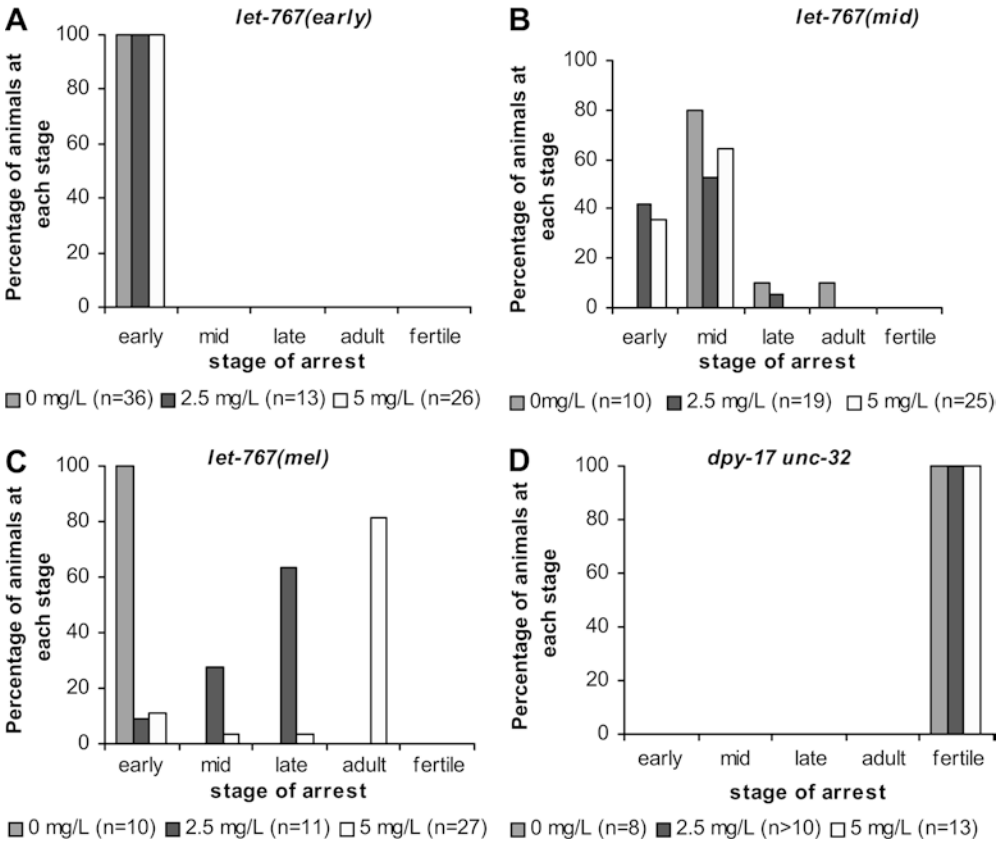



**Fig. 3** Effect of cholesterol removal on animals bearing *let-767* alleles. Dpy-Unc homozygous *let-767* animals were scored for arrest stages after 7 days of growth. Animals were grown either in the absence of cholesterol (*lightly shaded bars*), in the presence of 2.5 mg/l cholesterol (*dark bars*) or in the presence of the standard amount of cholesterol (5 mg/l) in the medium (*unshaded bars*)

In addition to the molting defects that are observed in *let-767* mutants, *let-767(mel)* adult hermaphrodites are approximately half the size of *dpy-17 unc-32* control animals (Fig. 4E–H). In addition to being smaller, *let-767 (mel)* mutants develop much more slowly than *dpy-17 unc-32* adult control hermaphrodites, reaching the adult stage 3–4 days later than *dpy-17 unc-32* animals ($n > 50$).

### *let-767 (mel)* mutants exhibit reproductive defects

The weak *let-767* mutant was assayed for proper germline formation and vulval development. Non-lethal adult hermaphrodites have a fully developed vulva and a fully developed germline that contains large oocytes and embryos near the vulva (Fig. 5A). In contrast to control animals, *let-767 (mel)* mutants display two distinct gonadal defects. Firstly, vulval development proceeds normally in *let-767 (mel)* animals but greater than 15% of adult *let-767* homozygotes have an extruding vulva phenotype (Evl) ($n > 100$) (Fig. 5B). Secondly, germline formation appears to be delayed, as approximately 20% of *let-767* adult hermaphrodites have a fully developed vulva, but a primitive gonad lacking any oocytes or embryos, although ultimately these animals do produce a few oocytes that are successfully fertilized (Fig. 5C). Non-lethal *dpy-17 unc-32* animals at the same stage have a fully developed gonad and vulva with embryos present in the uterus (Fig. 5A). *let-767(mel)* adult hermaphrodites contain germ cells that are morphologically similar to sperm, but rarely contain any oocytes–indicating a defect in oogenesis (Fig. 5D).

Due to the small size of *let-767(mel)* homozygotes, it is difficult to determine the extent of somatic gonad abnormalities. However, the gonads of this mutant are able to produce the limited number of fertilizable oocytes observed in *let-767(mel)* homozygotes. In about 10% of the *let-767(mel)* mutants somatic gonads were clearly visible; all of them were properly reflexed, but they were thinner and smaller than wild-type gonads ($n = 10$) (data not shown).

Furthermore, *let-767(mel)* mutants show a severe reduction in fecundity. The majority of *let-767 (mel)* mutants reach adulthood but produce only a limited number of embryos (7–10), which either develop into fully differentiated worms with a pharynx and a properly formed gut (~70%) (Fig. 5E) or appear severely disorganized and undifferentiated (~30%) (Fig. 5F).

## Discussion

In this paper we have demonstrated an essential role for a steroidogenic enzyme during *C. elegans* development. *let-767* mutants exhibit many developmental defects including slow and retarded growth, reproductive and molting defects, and defects in embryogenesis.

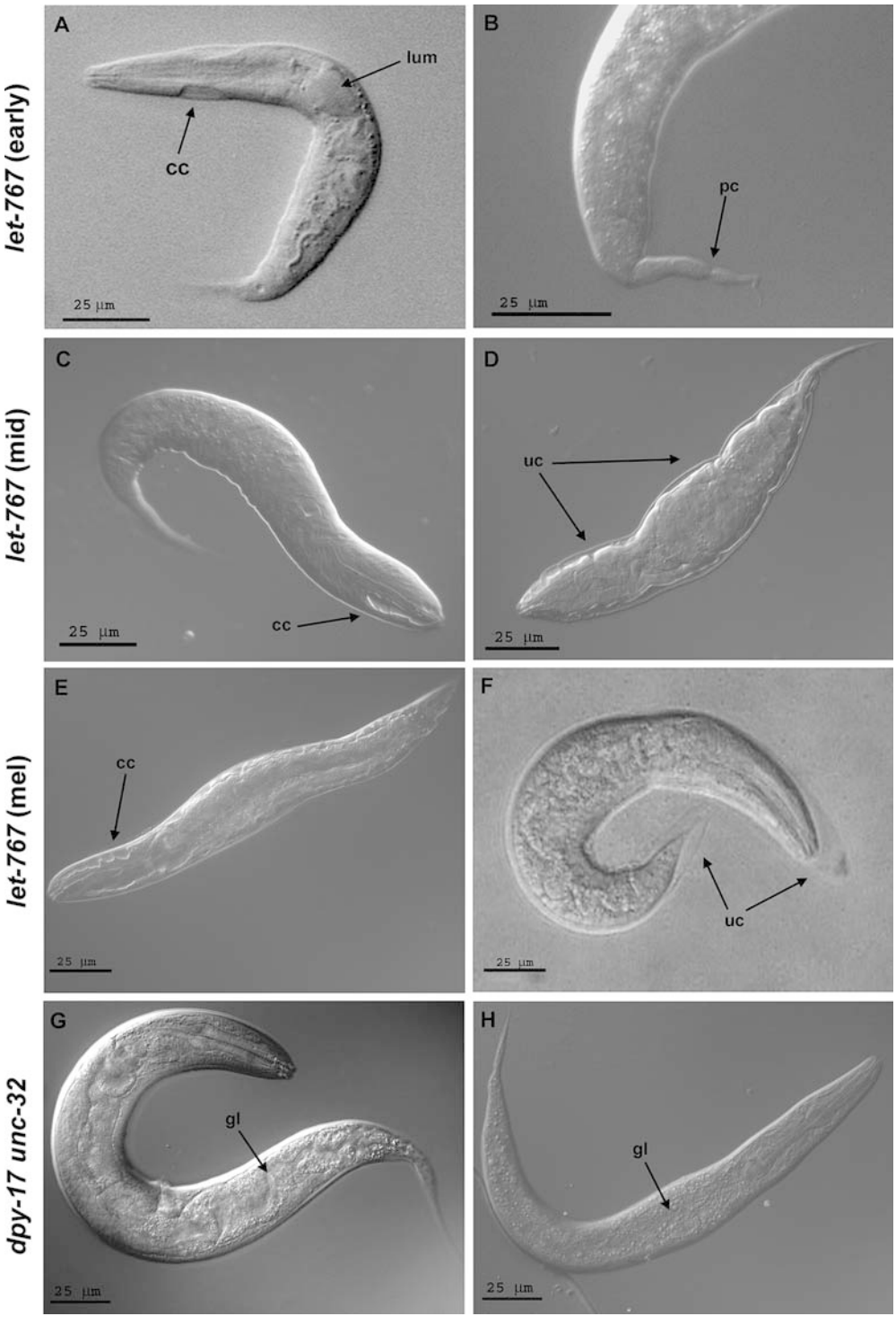
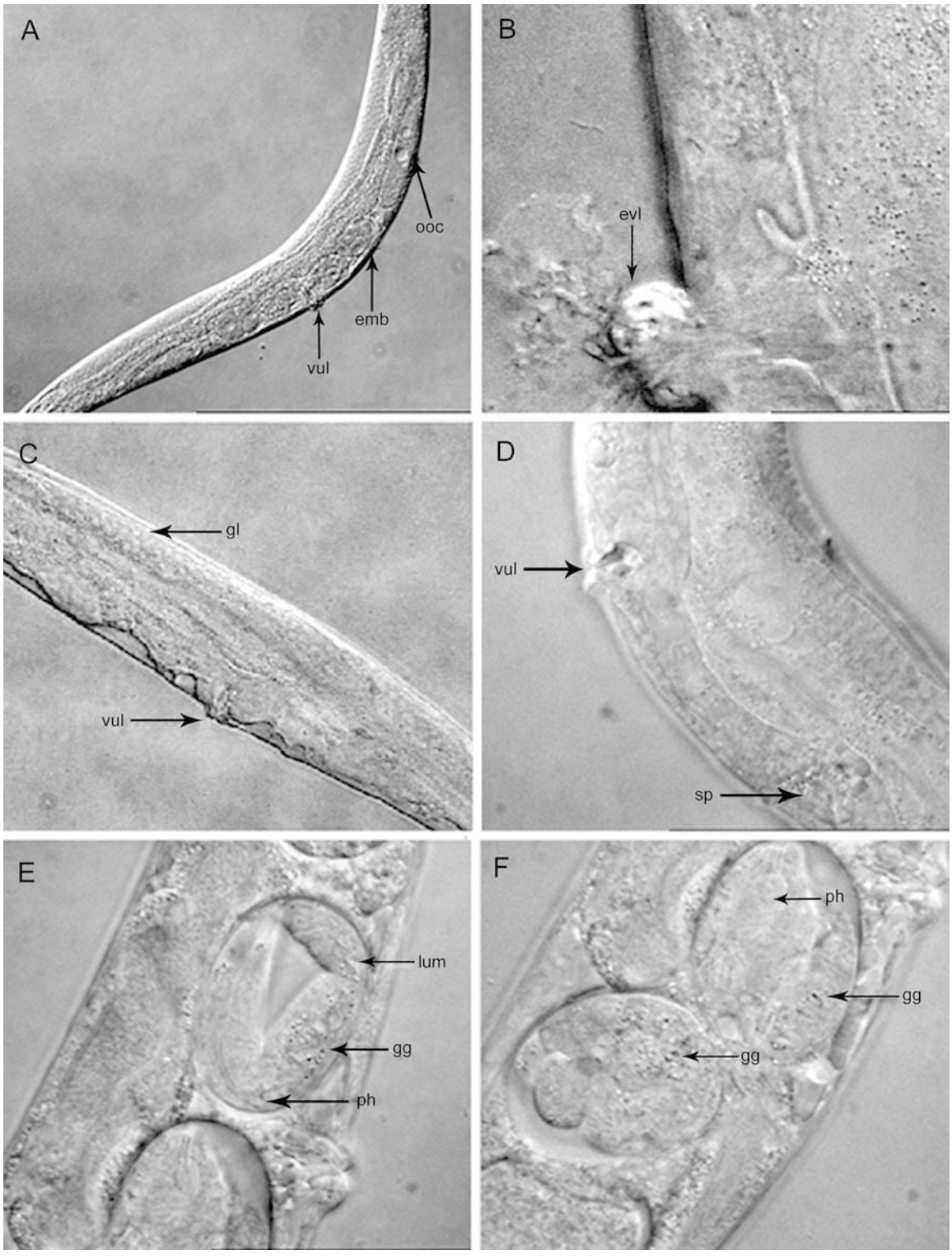



**Fig. 4A–H** Molting defects of *let-767* mutants. **A, B** *let-767 (early)* homozygotes. The mutant in **A** shows constrictions of the cuticle (cc) at the anterior end that are indicative of incomplete shedding of cuticle at the previous molt. The mutant in **B** has a constriction of the cuticle near the posterior end (pc, pinched cuticle). Panels **C** and **D** show *let-767(mid)* homozygotes. **C** Cuticle constrictions (cc) similar to those seen in *let-767 (early)* animals. **D** Points of attachment of an unshed cuticle (uc). Panels **E** and **F** show *let-767 (mel)* homozygotes. **E** Cuticle constrictions (cc) similar to those of *let-767 (early)* and *let-767 (mid)* homozygotes. **F** Unshed cuticle (uc) from a previous molt in *let-767 (mel)* homozygotes. **G, H** Non-lethal *dpy-17 unc-32* control animals. Panels **F** and **G** both show nongravid adult hermaphrodites. gl = gut lumen

*let-767* is expressed in the gut of larval and adult animals. The digestive tract of *C. elegans* is divided into four distinct sections: the anterior pharynx, derived from the ABa lineage, the posterior pharynx, derived from the MS lineage, the gut, derived from the E lineage, and the rectum, derived from the ABp lineage (Sulston and Hodgkin 1988). The specific expression of *let-767* within the E lineage of the digestive tract is reminiscent of that of the *ges-1* gene, which shares similar 5′ regulatory sequences with the *let-767* promoter. The expression of *let-767* in the gut is also compatible with the distribution of cholesterol in the gut (Merris et al. 2003). Further evidence for a role of LET-767 in sterol modification is the enhancement of the phenotype associated with weakest allele of *let-767* that is observed when the level of available cholesterol is reduced. A reduction in




growth and a decrease in brood size are phenotypes that are common to the *let-767* mutants and wild-type worms grown without cholesterol (Merris et al. 2003). In a previous study molting was reported to be disrupted in wild-type animals upon cholesterol deprivation (Yochem et al. 1999), but this was not observed in a more recent study (Merris et al. 2003). The latter authors seeded bacteria in cholesterol-free medium to ensure the complete absence of cholesterol in the medium (Merris et al. 2003), whereas this step was not reported by Yochem et al., and therefore a small amount of cholesterol may have been present the media used in this study (Yochem et al. 1999). Small amounts of cholesterol were present in our study and therefore it is pos-

**Fig. 5A–F** Phenotypes of *let-767 (mel)* adult hermaphrodites. Panel **A** shows a non-lethal *dpy-17 unc-32* control hermaphrodite with a normal complement of oocytes (ooc), a mature vulva (vul) and embryos (emb) in the uterus. **B** The everted vulva (Evl) phenotype seen in a fraction of *let-767(mel)* homozygotes. **C** Fully formed vulva (vul) accompanied by incomplete germline (gl) formation (absence of oocyte production). This animal also has the remnants of a previous molt seen as cuticle constrictions. **D** The presence of a mature vulva (vul) in an adult hermaphrodite indicates the capacity for production of mature sperm (sp). **E** Note the fully differentiated embryo containing a properly formed gut including a lumen (lum), gut granules (gg) and a pharynx (ph) in the uterus of a *let-767(mel)* homozygous hermaphrodite. **F** Note the presence of a disorganized embryo with some evidence of gut differentiation, as indicated by the presence of gut granules (gg) and a pharynx, in the uterus of a *let-767(mel)* homozygous hermaphrodite

sible that a reduction in the level, but not the complete elimination, of cholesterol results in a molting defect.

Although a direct role for LET-767 in molting and reproduction has not been demonstrated, it is tempting to speculate that the enzyme may be involved in the modification of a steroid that is required for these processes during the development of *C. elegans*. 20-Hydroxyecdysone is the molting hormone in insects, and is also required for embryogenesis, female germline formation and molting (reviewed by Kozlova and Thummel 2000). Due to a lack of evidence for ecdysteroid synthesis in *C. elegans* (Chitwood and Feldlaufer 1990), coupled with the lack of the ecdysone receptor genes within the *C. elegans* genome (Sluder et al. 1999), it is likely that processes, such as molting and reproduction, that are regulated by ecdysone in Drosophila are regulated by a different steroid hormone in *C. elegans*. As a result of the apparent divergence between these two species in the hormonal regulation of molting and reproduction, relatively little is known about the hormonal control of these processes in *C. elegans*. The implication of LET-767 in the steroidogenic processing of a *C. elegans* hormone involved in molting, growth and reproduction provides the opportunity to test this hypothesis more directly.

A link between Drosophila's ecdysone response and *C. elegans* has been demonstrated by the conservation of function of two orphan nuclear hormone receptors. βFTZ-F1 is an orphan nuclear receptor in Drosophila that is directly induced by another orphan nuclear receptor, DHR3, which in turn is directly induced in response to ecdysone. The *C. elegans* orthologues of these receptors, NHR-23 (DHR3) and NHR-25 (βFTZ-F1), are both required for reproduction, molting and embryogenesis in *C. elegans*. However, unlike βFTZ-F1, *nhr-25* is not directly induced by *nhr-23* (Kostrouchova et al. 2001), indicating a divergence in the hormone regulated pathway.

*let-767* mutants share similar phenotypes with *nhr-23* and *nhr-25*-mutants in embryogenesis, molting, and female reproduction. The earliest stage in development that requires *let-767*, *nhr-23* and *nhr-25* is embryogenesis (Kostrouchova et al. 1998; Asahina et al. 2000; Gissendanner and Sluder 2000). A deletion within the *nhr-25* locus causes embryos to arrest at the 1.5 to 2-fold stage of development with a disorganized structure and the presence of gut granules; this phenotype is similar to that of embryos derived from older *let-767(mel)* mothers (Asahina et al. 2000). The first embryos that are produced by *let-767(mel)* mothers are fully formed embryos that appear ready to hatch. It is likely that these embryos do not hatch due to an inability to shed cuticle. A failure to shed the cuticle completely at different stages throughout development is a phenotype that is shared with animals that are deficient in *nhr-23* function (Kostrouchova et al. 2001).

Observation of animals carrying the GFP fusion construct indicates that *let-767* is not expressed in the embryo. Therefore, this may indicate that fully functional LET-767 protein is deposited into the embryos. A similar situation exists for the vitellogenin proteins in adult *C. elegans* hermaphrodites. Vitellogenin is present at high levels in the gut of adult hermaphrodites, and is endocytosed by late stage oocytes via the vitellogenin receptor, RME-2 (Grant and Hirsh 1999). The maternal contribution of LET-767 protein is likely to be enough to allow the embryo to proceed to the first larval stage when the animal begins to transcribe *let-767* mRNA.

*let-767* plays a critical role in the hormonal control of female reproduction in *C. elegans*. Many aspects of female reproduction are disrupted in *let-767(mel)* mutants, including somatic gonad formation, vulva formation and oocyte production. Unlike wild-type animals that are grown under conditions of cholesterol limitation (Shim et al. 2002), *let-767 (mel)* adults do not contain multiple unfertilized oocytes. Interestingly, hermaphrodites that are deficient in either *nhr-23* or *nhr-25* function display significant defects in somatic gonad formation and vulva formation, including the everted vulva phenotype that is seen in animals that are deficient in *nhr-25* function (Asahina et al. 2000; Kostrouchova et al. 1998).

As a result of decreased oocyte production, the number of embryos produced by *let-767(mel)* homozygotes amounts to only 5% of the brood size produced by wild-type hermaphrodites, indicating a role for *let-767* in fecundity. Inefficient oocyte production may be the sole cause of the reduced fecundity that is observed in *let-767(mel)* mutants. In wild-type animals, oocytes are produced in an assembly-line fashion along the gonad arm where the most proximal oocyte is used for fertilization. Prior to fertilization and passage through the spermatheca, oocytes become larger due to uptake of the yolk proteins that are secreted into the oocyte by the gut. Failure to transfer these yolk proteins would result in a decrease in oocyte production and therefore a decrease in fecundity. This situation exists in Drosophila females that are mutant for the ecdysone receptor EcR. EcR mutants have severe reductions in fecundity due to a failure to accumulate yolk protein in the oocytes and other defects in oogenesis (Carney and Bender 2000). LET-767 may be required to modify a hormone that is

required for the process of yolk protein uptake by late-stage oocytes. Enough LET-767 protein may be present in *let-767(mel)* mothers to produce the hormone that is required for fully functional oocytes, resulting in the production of a few normal embryos. However, as the contribution of yolk deposits is reduced due to the (relative) lack of LET-767, fertilized embryos do not contain enough nutrients to undergo morphogenesis properly, and therefore arrest as disorganized embryos within the uterus. A further reduction in yolk protein deposition would result in the failure of oocyte production that is seen in *let-767(mel)* mothers. Therefore, LET-767 may modify a hormone, either within the digestive tract or within the germline, that is required for oogenesis and vitellogenesis.

The hormonally regulated pathway(s) that control(s) proper development and reproduction in *C. elegans* is beginning to take shape. *nhr-23* and *nhr-25* play related roles during development to regulate molting and reproduction, but appear to do so in separate pathways. *dpy-7,* which encodes a cuticle collagen, has been placed downstream of *nhr-23* (Kostrouchova et al. 2001). LET-767 may modify a steroid hormone that is directly upstream of *nhr-23* or *nhr-25* and is required to induce the expression of genes that are required for molting and reproduction.

Recent studies in Drosophila have implicated the product of the *dare* gene as a steroidogenic enzyme that is involved in the modification of ecdysteroid derivatives (Freeman et al. 1999). It will most certainly be very interesting to determine whether the Drosophila orthologue of LET-767 plays a role in the hormonal regulation of molting and reproduction. In any case, it is clear that LET-767 plays a critical role in many aspects of development, including molting, reproduction, and embryogenesis.

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