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A characterization of the gene omega : a modifier of a cuticle protein in Drosophila melanogaster

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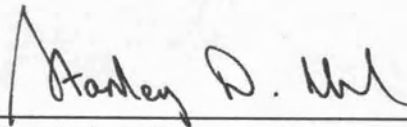
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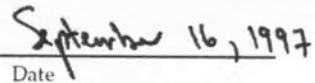
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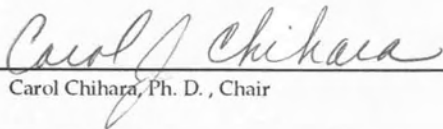
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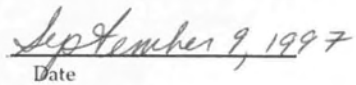
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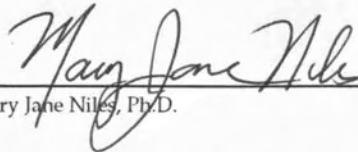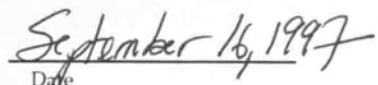
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Carol Chihara, Ph. D. , Chair

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Mary Jane Niles, Ph.D.

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Date   September 16, 1997

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A Characterization of the gene *omega*: a Modifier of a Cuticle Protein in
Drosophila melanogaster

A Thesis presented to the Faculty of the Department of Biology,
University of San Francisco
in partial fulfillment of the requirements for the Honors degree in Biology.

By

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September 10, 1997

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Acknowledgments

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Abstract

omega+ is a modifier of the LCP5 and related genes. Evidence indicates that OMEGA is a dipeptidylaminopeptidase (DPAP) cleaving its substrate at the amino terminus after R-P. The EMS induced recessive form of *omega* fails to cleave the Arg-Pro dipeptide changing the migration pattern of LCP5 (and related proteins) in PAGE. Microsequencing the N-terminal residues of Canton S wild type LCP6, LCP7, LCP8, LCP9 and another EMS induced mutant RHO indicate that LCP6 and RHO represent alternate and mutated copies of LCP5, respectively. LCP6 demonstrates a shift in migration pattern characteristic of LCP5 related cuticle proteins; however, a point mutation in RHO substituting serine for proline in the penultimate residue eliminates the *omega* DPAP recognition site and subsequent hydrolysis. Furthermore, LCP8 matches the translated sequence of *dcp8*, a recently sequenced gene in the 65A5-6 cuticle protein cluster. LCP7 and LCP9 are not present in either known cuticle protein gene cluster (65A5-6 and 44D). *omega* has been observed to have two pleiotropic effects: a 24H delay prior to pupariation and a specific reduction in male fertility of 33% in *omega* homozygote males as compared to wild type males. It is probable that the developmental delay and the male specific reduction in fertility are tied to the *omega* gene product's failure to act as a DPAP.

Introduction

The study of the regulation of eukaryotic gene expression is integral to understanding the development of a single celled zygote into a multicellular organism. The cells at each stage of development can be characterized by their individual complements of expressed gene products (Wade, 1981). This molecular characterization of gene expression during normal development may aid in discerning the errors which culminate in some developmental genetic disorders and unregulated cell proliferation.

Since its introduction as a subject for genetic analysis in 1906 *Drosophila melanogaster* has proven to be an ideal organism. The 12 day lifecycle, many easily identifiable phenotypes, availability of multiple strains and ease of manipulation and culture were in the past sufficient reasons to study genetics in Drosophila. As a result of that early work, the vast amount of information about the genetics and biology of *Drosophila* has increased its appeal as one of the developmental geneticist's organisms of choice.

The urea soluble cuticle proteins of *Drosophila melanogaster*, in particular, are an excellent system for analysis of hormonally regulated and mostly clustered genes which are expressed in a developmentally regulated pattern throughout development (Hodgetts *et. al.* 1977; Chihara *et. al.* 1982). Many developmentally important genes in the insect are initially regulated by the steroid hormone 20-hydroxyecdysone (Ashburner 1990 for review). *E.g.* there is evidence that the cuticle protein genes of the German cockroach, *Anthonomus grandis* and the boll weevil, *Blattella germanica* are among those regulated by ecdysone levels (Stiles and Newman 1992). Similarly, the *Drosophila melanogaster* pupal cuticle proteins have been shown to be regulated by ecdysone pulses in *in vitro* imaginal disk cultures (Doctor, *et. al.* 1985). Recently, the molecular mechanism for steroid gene induction has been elucidated.

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Ecdysone facilitates dimerization of the proteins EcR (ecdysone receptor) and USP (Ultraspiracle). The resulting heterodimer binds to DNA sequences termed ecdysone response elements (EcREs) (Yao *et. al.* 1993; Antoniewski *et. al.* 1995) which when bound regulate transcription.

The development of *D. melanogaster* at 25°C proceeds from egg deposition to a sexually mature adult within 10-12 days. Within 24h of fertilization, the first instar hatches from the egg. Two 24h larval stages follow, during which the larva feeds while burrowing in the media. The third instar lasts two days, two of which are similarly spent feeding. At some point prior to pupariation, the larva crawls out of the media and wanders searching for a suitable place on which to pupariate. Within four to five days of pupariation, the adult fly emerges from the pupa case and is soon ready to mate (within six to eight hours). As the insect grows its external 'skeleton' (the cuticle) must be molted and a new, larger cuticle must be deposited. Molts are presumed to be precipitated by pulses of high levels of 20-hydroxyecdysone.

Cuticle serves as an insect's exoskeleton, providing both anchoring points for muscle and protection from the environment. Generally, insect cuticles are composed of chitin (a polymer of N-acetyl-glucosamine), waxes, lipids and protein (Elzinga, 1987). Chihara *et.al.* (1982) demonstrated that there are four distinct *D. melanogaster* urea soluble cuticle protein patterns for the five life stages (the first and second instars sharing a similar protein pattern). The third instar facilitates cuticle protein study because a single isolated third instar cuticle contains sufficient protein to be visualized by Coommassie blue staining after non-denaturing polyacrylamide gel electrophoresis (PAGE) following urea extraction.

There are five major proteins (LCP1-5) and five minor proteins (LCP6-9, & LCP2a) visible by PAGE when a third instar single cuticle is extracted with urea

Figure 1: Schematic of 15% PAGE separation of third instar cuticle proteins. The major bands are depicted as the darkest bands (1-5). The *omega* protein banding pattern is characterized by the appearance of a band between LCP3 and 4, and a lack of a band at position 5. The arrow indicates the position of the 5 band absent in the *omega* mutant. *rho* is characterized by the appearance of a band between LCP3 and 4 shifted above *omega*. An actual stained gel can be found in Chihara and Kimbrell (1982).
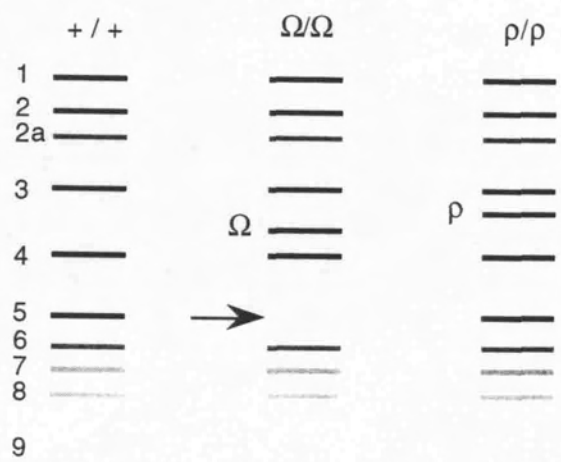
(figure 1; nomenclature as Chihara *et.al.* 1982). The genes coding for LCP1-4 have been localized to a 7.9 kb cluster on the second chromosome at 44D (Snyder *et.al.* 1982). Transcribed divergently, their organization may contribute to their regulation. Another cuticle protein cluster has been identified at 65A5-6 (position 11 on the third chromosome [Charles *et.al.*, in press; Chihara and Kimbrell 1986]). In this case approximately 22kb of genomic DNA was sequenced and twelve genes and/or pseudogenes were identified. Again the genes are organized in two divergently transcribed clusters, this time separated by a 4.5 kb spacer. The genomic DNA sequenced came from *D.melanogaster* iso-1 strain and contained two copies of *lcp5* and three copies of *lcp8* . Hybridization of clones to restriction digested Canton S DNA suggested the presence of three copies of *lcp5* in that particular strain. Mapping indicates that also included in the third chromosome cluster are Oregon R *lcp6* and an ethane methane sulfonate (EMS) induced mutant *Rho* (Chihara and Kimbrell 1986). Analysis of LCP6 and RHO N-terminal residues indicates that these proteins are coded for by modified copies of *lcp5* (See Results and Discussion).

Rho is a codominant mutant producing a protein band that migrates between LCP3 and 4 (figure 1). Another EMS induced mutant *omega* is a recessive modifier of LCP5, shifting its migration pattern in PAGE (figure 1). Wild type LCP5 follows LCP4, while LCP5^{omega} migrates between LCP3 and LCP4 slightly below RHO. *omega* has been shown to map at ca. 40.43, 29.3 map units way from the 65A5-6 gene cluster (Schneider 1990). Based upon this data deficiency mapping was performed and indicated that *omega* lay at approximately 69 (Schneider 1990). However, further deficiency mapping and new cytology provided for the deficiencies previously tested revealed the need to update *omega*'s cytology (see Results and Discussion).

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Previous observations indicated that the *omega* stock was developmentally slow in one of the three larval instars by about 24h and that *omega* males exhibited a reduced fertility when compared to wild type (Chihara, personal communication).

This thesis presents work further characterizing third instar cuticle proteins and *omega*, the recessive modifier of LCP5.

Materials and Methods

Stocks

Fly stocks and crosses were maintained at 25°C on standard cornmeal-molasses-yeast-agar media containing Tegosept and proprionic acid and supplemented with live yeast in half-pint bottles or vials.

"Wild type" designation is given to both a strain of Oregon R *Drosophila melanogaster* maintained under laboratory conditions for at least 20 years at the University of San Francisco and its third instar cuticle protein electrophoretic pattern.

omega (Ω) and *Rho* are ethane methane sulfonate induced mutants previously described by Chihara and Kimbrel (1986). These stocks have been maintained under laboratory conditions at the University of San Francisco for at least 10 years.

Canton Special (Canton S) "wild type" strain used for micro sequencing of LCP7, 8, and 9. Canton S was obtained from Dr. Lynn Riddiford, University of Washington.

Balanced third chromosome deficiency stocks were used to determined the location of *omega* using the cytological breakpoints of the deletions (See Table 1):

·*D-5rv5, D-5rv12, D-5rv14*are deficiency stocks obtained from Dr. Adelaide Carpenter, University of Cambridge.

00732 and *st-f13* are deficiency stocks obtained from Amy Beaton in the lab of Dr. Gerry Rubin, University of California, Berkeley.

Brd6, Bk10, fz-M21 are deficiency stocks obtained from the Bloomington *Drosophila* Stock Center.

LE392 is a supressing strain used to propogate bacteriophage λ. Its genotype is *supE*44, *supF*58, *hsdR*514, *galK*2, *galT*22, *metB*1, *trpR*55, *lacY*1. It was used to prepare lysates of lgt10 cDNA clones.

omega crosses for fertility analysis

Five virgin *omega* female flies were placed in the same vial with a single *omega* male for five days at which point the females were separated into individual vials and the male discarded. The offspring in all six vials (the original vial and the five individual vials) were scored for eleven days following the emergence of the first adult fly. Ten replicates of this virgin female Ω/Ω x Ω/Ω cross were performed. The same crossing scheme was performed for the following pairings:

<div align="center">

virgin female $+/+$ x Ω/Ω male

virgin female Ω/Ω x $+/+$ male

virgin female $+/+$ x $+/+$ male

virgin female $\Omega/+$ x Ω/Ω male

virgin female $\Omega/+$ x $+/+$ male

</div>

Data was compared using a single tailed T test on Microsoft Excel 5.0.

omega modification of Oregon R "wild type" L3CP6

Virgin *omega* female flies were crossed to wild type males. The offspring males were back-crossed to virgin *omega* female flies. The back-cross third instar offspring were dissected and run on nondenaturing gels as described in Chihara *et. al.* (1982). Gel controls included wild type, *omega* homozygous, and heterozygous third instar cuticles. See figure 5 for crossing scheme.

Deficiency Mapping

omega virgin females were mated to deficiency stocks to determine the cytological location of *omega*. *omega* was tested with those deficiencies described above. F_1 larval cuticles were hand dissected in *Drosophila* Ringer's Solution (1.89 mM $CaCl_2$, 4.7 mM KCl, 128.4 mM NaCl). Cuticle proteins were extracted

from individual larvae in 20μl of urea extraction buffer (7 M urea, 5 mM

Tris•HCl pH 8.6, 7% β-mercaptoethanol and enough bromophenol blue to serve

as tracking dye). Vertical gel electrophoresis was performed in a 0.75 mm slab gel

(15% acrylamide, 0.08% bis-acrylamide, 0.375 M Tris•HCl, pH 8.6). Note that

while there are no components of the gel that are denaturing, the cuticle proteins

are loaded directly onto the gel in the 7M urea extraction buffer. At least eight

progeny larvae were dissected per cross to reduce the probablility of missing the

homozygote *omega* to 1/256. Gels were stained in 0.01% Coomassie G-250, 10%

acetic acid, 5% methanol and destained in 10% acetic acid, 5% methanol.

Protein Microsequencing

Third instar larvae cuticles were prepared in batches of 50 cuticles from

wild type, *omega* and *Rho* by whirling in a Waring blender microcontainer

(Eberbach) for 2 minutes in cold Ringer's (1.89 mM $CaCl_2$, 4.7 mM KCl, 128.4 mM

NaCl). Cuticles were extracted using urea extraction buffer (20μl/cuticle) and

separated using 15% non-denaturing 1.5 mm preparative PAGE (slab gel formula

as above). Edges of preparative gel were cut and proteins visualized in

Coomassie stain. R_f values were calculated for proteins of interest (LCP5,

LCP5*omega*, RHO, LCP7, LCP8, LCP9) and the appropriate center gel section was

prepared for semi-dry blotting.

A semidry transfer procedure was done according to LeGendre (1993).

Immobilon-P^SQ Transfer Membrane (Millipore ISEQ15150) and 6 VWR 238

Blotting paper sheets (Whatman 3MM equivalent) per gel were cut to the same

size as gel. Three blotting paper sheets were soaked in Cathode Buffer (25 mM

Tris, 40 mM glycine, 10% methanol, pH 9.4), one in Anode Buffer I (0.3 M Tris,

10% methanol, pH 10.4) and two in Anode Buffer II (25 mM Tris, 10% methanol,

pH 10.4). The cut membrane was wet in 100% methanol for 1-3 seconds, then

immersed in Milli-Q water for 2-3 minutes and finally equilibrated in Anode Buffer II for at least 15 minutes. Meanwhile, the gel was equilibrated in Cathode Buffer for 10 minutes. The transfer stack was assembled on the anode plate in the following order: blotting paper soaked in Anode Buffer I, blotting paper soaked in Anode Buffer II, membrane, equilibrated gel, and blotting paper soaked in Cathode Buffer. The transfer was achieved by the application of constant current (10.0 mA per cm^2 of gel) for at least 3 hr. After transfer, the membrane was rinsed in Milli-Q H2O 2-3 times for 5 minutes each wash at room temperature. The washed membrane was stained in Coomassie R-250 (0.1% in 50% methanol) for 2 minutes and destained with several changes of 50% methanol, 10% acetic acid. Destain was followed by several rinses of Milli-Q water. Lastly, the membrane was air dried and stored in a sealed plastic bag at -20°C until hand delivered to microsequencing laboratory.

Proteins were microsequenced at the Biomolecular Resource Center, University of California, San Francisco.

Southern Blot of Putative LCP9 Clones

A Southern blot of seven (λ1-λ6,λ8) λgt10 third instar cDNA clones was performed using a digoxigenin (DIG) labeled degenerate oligonucleotide probe (see figure 2) based upon 10 N terminal amino acid residues from LCP9 using Boehringer Manheim Genius System.

3′ Tailing of LCP9 Degenerate Oligonucleotide

The degenerate lcp9 oligos were labeled using Boehringer Mannheim Genius System, version 2.0, Kit 6 and the suggested procedure. The following reagents were added to a microfuge tube on ice in the following order:

5X Genius Labeling Reaction Buffer	4 μl
25 mM CoCl2 solution	4 μl
DIG labeled dUTP	1 μl
degenerate oligonucleotide	5 μl (1 μg/μl)
dATP	1 μl
Terminal Transferase	1 μl
dH2O	4 μl

Both the experimental and the provided unlabled oligonucleotide reactions were incubated at 37°C for 15 minutes. The respective tubes were placed on ice and 1 μl glycogen (20mg/ml) and 1 μl 200 mM EDTA, pH 8.0, solutions were added. The DNA was precipitated with 0.1 volume 4M LiCl and 2.5-3 volumes ethanol (-20°C); the solution was then vortexed and incubated at -70°C for 30 minutes. The microfuge tube was centrifuged at 14krpm, 4°C for 15 minutes and the ethanol was removed by vacuum aspiration. The precipitate was washed with 100 μl of 70% ethanol at 4°C and then followed by centrifugation at 14krpm, 4°C for 5 minutes. Again the ethanol was aspirated away and the pellet was dried and stored in 20 μl TE/0.1% SDS at -20°C.

Estimating Yield of Labeled Oligonucleotide

Serial dilutions (1:10, 1:100, 1:1000, 1:10,000) were made of the experimental labeled oligo, control labeled oligo, and a pre-labeled control supplied in Genius Kit 6. 1 μl of each dilution was spotted onto a nitrocellulose membrane (Gelman Sciences Biotrace NT) and the membrane was baked at 80°C for 30 minutes. The following incubations were performed at room temperature each in heat sealed plastic bags:

Genius Buffer 1 (100 mM Tris•HCl, 150 mM NaCl, pH 7.5 filtered through 0.45 μm filter) 30 seconds.

Genius Buffer 2 (2% [w/v] blocking reagent in Genius Buffer 1)- 5 minutes.

Conjugate antibody solution (1:5000 dilution of a dig-alkaline phosphatase in Genius Buffer 2)- 5 minutes

2X in Genius Buffer 1- 5 minutes per wash.

20 ml of Genius Buffer 3 (100mM Tris•HCl, pH 9.5, 100mM NaCl, 50mM MgCL2)- 2 minutes.

Color Substrate Solution (45 µl NBT solution, and 35µl x-phosphate solution in 10ml Genius Buffer 3 without agitation in the dark ≤ 12 hrs.

2X dH2O- 5 minutes.

Labeled experimental oligo concentration was determined by comparing spot intensities between experimental oligo and provided labeled control oligonucleotide. The membrane was dried and placed in a heat sealed plastic bag for storage.

Bacteriophage Preparation

100µl of λgt10 isolated phage containing third instar cDNA were mixed with 300µl of an over-night culture of LE392 and incubated at 37°C for 20 minutes. After incubation 3 ml of LB top agarose was added and media was poured on pre-warmed LB agar plates. Plates were allowed to harden upright at room temperature, then incubated inverted over night at 37°C. 3 ml of λ diluent (10 mM Tris•Cl, 10 mM MgSO₄, pH 7.5) was added to each plate. The plates were then incubated under constant gentle agitation for 3 hrs at which point the λ diluent was transferred to 15 ml tube. Each plate was washed with 2 ml of λ diluent; each wash was then transferred to appropriate 15 ml tube. 20µl of chloroform was added to the wash tube followed by centrifugation at 2000 rpm at 4°C for 25 minutes in an IEC centrifuge to remove bacterial contamination. Supernatant was transferred to a fresh 15 ml tube; 20 µg of RNase/DNase were added and the tubes were incubated for 30 minutes at 37°C. Each sample was

aliquoted in 750μl portions into 1.5 ml microfuge tubes. Equal volume of 20% PEG (8000), 2 M NaCl in SM (0.1 M NaCl, 10 mM $MgSO_4$, 50 mM Tris•Cl, pH 7.5, 0.01% gelatin) were added to each tube. The samples were incubated on ice for 1-3 hrs. This incubation was followed by centrifugation at 14000 rpm for 10 minutes at 4°C. The supernatant was removed by vacuum aspiration, the samples were air dried for at least 10 minutes, and the pellets from each clone were pooled and re suspended in 0.5 ml TE (10 mM Tris•Cl, 1 mM EDTA, pH 8.0).

Uncut λ samples were electrophoresed along with partially BamHI digested *Drosophila melanogaster* genomic DNA in a 0.8% agarose 1X TBE mini-gel (see figure 3) at 90V for 1 hr. DNA was electroblotted onto a nitrocellulose membrane that had been wet in 1X TBE with VWR 238 Blotting paper (Whatmann 3MM equivalent) also soaked in 1X TBE at 100mA for 3 hrs under constant current. DNA was fixed onto a membrane by baking at 87°C for 2 hrs.

Hybridization of 3' Tailed oligonucleotide proceeded over night as specified by Boehringer Mannheim at 54.2°C. In hybridization buffer (5X SSC, 1% Blocking reagent in Genius Buffer 1, 0.1% N-lauroyl sarcosine, 0.02% SDS, labeled oligonucleotide) 100μl (10 mg/ml) poly A and 100μl (500 μg/ml) poly dA were included. Detection was also performed as specified by manufacturer in heat sealed plastic bags.

Drosophila melanogaster Genomic DNA Preparation

50 adult Oregon R wild type animals were homogenized in homogenization buffer (0.1 M Tris•Cl, pH 9.1, 0.1 M NaCl, 0.2 M sucrose, 0.5 M EDTA, 0.5% SDS). The sample was divided into two microfuge tubes. To each 25 μl RNase (10 mg/ml) was added, followed by room temperature incubation for 30 minutes and a 30 minute incubation at 65°C. 75 μl of 8M potassium Acetate

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was added, the samples were vortexed and then incubated on ice for 30-60 minutes. The samples were centrifuged at 14000 rpm for five minutes at 4°C. The supernatant was transferred to clean microfuge tube. They were then phenol:chloroform (1:1) and chloroform extracted. The genomic DNA was precipitated with 1 volume 95% ethanol at room temperature. The supernatant was vacuum aspirated and the precipitate was washed with 70% ethanol at 4°C. The DNA was re suspended in 50µl TE and stored at -20°C. DNA was quantitated using UV spectrophotometer.

Genomic DNA Digestion

2 µg of genomic DNA was digested in a final volume of 30 µl with 1 µl of BamHI overnight at 37°C.

Results and Discussion:

Initial work with *omega* included microsequencing the N terminal residues of LCP5^{+} and LCP5[*omega*] (see figure 2; Mandalaparthy, *et.al.* 1996; Schneider 1990). Preliminary data led to the thought that the *omega* mutant product modified LCP5 through the addition of some functional group during the normal protein processing for a secreted product. An EMS induced mutant, *omega*'s mutant phenotype is most likely due to a point mutation. This has been substantiated by the occurence of a relatively rapid stock reversion to wild type (Chihara, personal communication). The wild type OMEGA, therefore, acts post-signal peptide removal as a dipeptidylaminopeptidase (DPAP) cleaving the fifth cuticle protein and its variants, along with other as yet unidentified substrates, C-terminal to R-P. Similar protein processing has been found in other organisms, both eukaryotic and prokaryotic, cleaving after X-P or X-A. (There is some evidence of cleavage, albeit with reduced efficiency, even after X-G.) In insects DPAPs have been shown to function in the biosynthesis of lytic and bactericidal peptides, for example, melittin and cecropins, respectively (Kreil, 1990). The pleiotropic effects of *omega* (see below) can therefore reasonably be rationalized as due to the reduced function of some unknown OMEGA substrate(s) caused by lack of OMEGA DPAP cleavage in the various tissues and developmental stages which express *omega*. Thus far, the N-termini of *omega* modified and unmodified LCP5 and LCP5[Sr] (see Chihara and Kimbrell, 1986) have been microsequenced and both exhibit the same *omega*[+] form of X-P cleavage (figure 2; Chihara, personal communication).

The major N terminal sequences obtained from the Biomolecular Resource Center are presented in figure 2. Very little can be said about LCP7, 8, and 9. LCP8 has the same predicted sequence as recently identified *dcp8* (Charles, *et. al.*, in press) and, therefore, belongs to the third chromosome cluster,

Figure 2: Micro sequencing data of the N terminal residues of third instar *Drosophila melanogaster* cuticle proteins. Protein sequences are aligned to demonstrate their identity. Note the extra residues included in the EMS induced *omega* and *rho* mutant proteins. In all cases the residues with the strongest detection signal is reported. LCP8 is a 100% match with *dcp8* contained in the 65A5-6 cluster (Charles *et. al.*, in press).

LCP5	NLAEIVRQVSDVEP
LCP5 *omega*	RPNLAEIVRQVSDVEP?K?S
RHO	RSNLAEIVRQ
LCP6	NLAEIVRQVS
LCP7	GVEVLRSDSN
LCP8	AAEEPTIVRS
LCP9	NEEADVVKSD

supporting meiotic mapping data obtained by Chihara and Kimbrell (1986). LCP7 and 9, on the other hand, do not match any known cuticle protein genes in either the third chromosome cluster (at 65A5-6) or the second chromosome cluster (at 44D) and may compose a separate cluster of cuticle proteins. In an effort to identify *lcp9*, Christian Wade screened a third instar cDNA library in λgt10 using a degenerate oligonucleotide probe based upon the N-terminal amino acids. His secondary screen resulted in eight clones. A southern blot was performed on seven of the eight clones using the digoxigenin poly-U end labeled degenerate *lcp9* oligo identifying clones λ1, λ2, and λ8 as containing *lcp9* -specific cDNA clones (see figure 3 and 4). Once isolated the *lcp9* cDNA will be used to identify the cytology of *lcp9*, by an *in situ* hybridization, and the gene sequence.

It is interesting to note that the N-terminus of LCP5 and LCP6 are identical and, as yet, the only apparent difference between RHO and LCP5[omega] is the substitution of a serine for a proline residue; the rest of the N-terminal RHO sequence is identical to LCP5. Looking at the gene sequence of *lcp5* presented in figure 7 of Charles, *et. al.* (in press) this amino acid substitution results from a CCC→TCC point mutation in the first nucleotide of codon eighteen (*lcp5* corresponds to *dcp3* in Charles, *et. al.*). That substitution of a serine for a proline may account for the *omega*[+] failure to modify RHO and subsequently its distinct migration pattern in PAGE.

In Chihara and Kimbrell (1986) rho was placed very close to lcp5; this new data argues that rho is actually an EMS mutated copy of lcp5 contained within the same third chromosome cluster. It has been shown that two D. melanogaster strains (iso I and Canton S) contain multiple copies of lcp5 in the 65A5-6 cluster (Charles,et al in press). It stands to reason that the multiply marked stock used to induce cuticle protein mutations also contained multiple *lcp5* copies.

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Figure 3: Seven clones from a secondary screen of a λgt10 third instar cDNA library were isolated and run on 0.8% agarose gel 1X TBE along with genomic *Drosophila melanogaster* Oregon R wild type DNA. The gel was stained with ethidium bromide prior to transfer and photographed. Note the large amount of RNA still present in the phage lysates and the poor digestion acheived by a 2hr BamHI incubation at 37°C. λ1-λ6, λ8 - clone designations, G - *Drosophila melanogaster* genomic DNA BamHI digest.
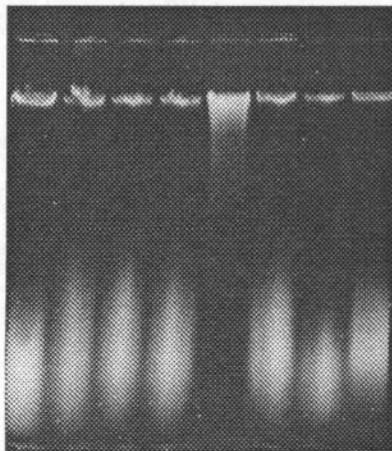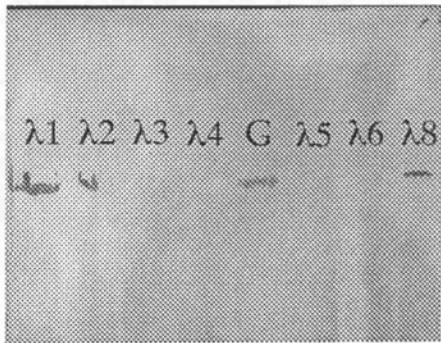
λ1 λ2 λ3 λ4 G   λ5 λ6 λ8

Figure 4: The nucleic acids present in the gel from figure 3 were transferred to membrane and then probed with a degenerate *lcp9* oligonucleotide probe based upon the ten N terminal amino acid residues of LCP9. For amino acid sequence see figure 2. λ1-λ6, λ8 - clone designations, G - *Drosophila melanogaster* BamHI digest.

λ1 λ2 λ3 λ4 G λ5 λ6 λ8

Furthermore, Oregon R wild type *lcp6* also represents a changed copy of *lcp5*. This is substantiated by the inability of Chihara and Kimbrell (1986) to isolate *lcp5* and *lcp6* by meiotic mapping, their identical N termini and by the *omega* modification of Oregon R wild type LCP6 in a Ω/Ω background. A modified PAGE migration pattern when placed in an *omega* homozygote background was previously used by Chihara and Kimbrell (1986) as a means of identifying *lcp5* variants. The offspring of the $\Omega/+ X \Omega/\Omega$ backcross have four genotypes with three readily identifiable phenotypes (see figure 5 and 6): wild type, LCP5*omega*, and LCP5*omega*, LCP6*omega*. This last phenotype represents the omega-lcp6 recombinant flies. The *omega -lcp6* recombinant banding pattern was seen in 3 of 23 dissected larvae, a recombination frequency of 0.13. Both the N terminal sequence identity and the shift in migration pattern of LCP6 when in an *omega* homozygous background suggests a structural/functional similarity between Oregon R LCP5 and LCP6. If *lcp6* is a homologue of the identified and sequenced *lcp5* , it is plausible that *lcp6* would be found in the 65A5-6 gene cluster as a duplication of *lcp5*. If so, the recombination frequency between *omega* and *lcp6* would be similar to the map distance between *omega* and *lcp5* (29.3 cM, Schneider, 1990). Chi Square analysis (p=0.33) indicates that the hypothesis is plausible if we assume that the three *omega -lcp6* recombinant larvae represent half of the recombination frequency. The recombination frequency is then 0.261.

Previous work had placed *omega* 29.3 cM from *lcp5* , as noted above. Based upon the most recent cytologies presented on Flybase *omega* has been localized to 70E8;71A1 by deficiency mapping (See figure 7 and Table 1). *omega* is uncovered by three deficiencies induced at three different laboratories: Df(3L)Brd6, gamma ray induced by J. Posakony; Df(3L)fz-M21, gamma ray induced by Adler; and Df(3L)D-5rv series X-ray induced by A. Carpenter (FlyBase as of May 28, 1997). The presented cytology for *omega* is dependent upon not only deletions which

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uncover *omega* , but also deletions which do not, in particular Df(3L)00732 and Df(3L)Brd15 and Df(3L)fzD21.

Once localized, the cytology will aid in identifying the *omega* locus, which should aid in its characterization. The DNA itself (and the translated amino acid sequence), however, will only provide partial explanations to a few of our questions such as the developmental delay mentioned earlier and the sex specific reduction in fertility. *omega* males demonstrate a reduction in fertility as compared to wild type males in all crosses performed (with *omega* homozygote females a two tailed student's t: $P = 7.7 \times 10^{-5}$, with wild type females: $P = 2.3 \times 10^{-6}$, heterozygote females: $P = 9.9 \times 10^{-3}$, where P is the probability that the samples are taken from the same population and therefore have equal means. Probabilities are based upon means presented in figure 8, assuming unequal variances; Microsoft Excel 5.0 for Macintosh). This reduced fitness in *omega* males is likely caused by the synergistic effect of two *omega* characteristics. First, *omega* homozygous males appear to be impaired in successfully copulating with females as compared to wild type males (see figure 9). Five days after introducing males to the females, the females were separated into individual vials and the males discarded. In crosses using *omega* homozygotes, it is clear that fewer successful matings occurred (see figure 10). Second, of those successful matings *omega* homozygote males sired fewer adult offspring. And this reduction cannot be attributed to a developmental problem associated with offspring possession of an *omega* allele (see below) and therefore must be associated with spermatogenesis. The successful copulation frequency (SCF) when an *omega* homozygote male is mated to an *omega* homozygote female is 0.62 (as compared to a wild type male mated to an *omega* homozygote female which has an SCF of 1.0). The reduction in adult offspring numbers expected from an *omega* male cross is given by the number of adult offspring from the appropriate control cross multiplied by the

Figure 5: *omega* modification of Oregon R LCP6 cross scheme and expected results. The *omega* flies' sixth cuticle protein variant is designated mms for "multiply marked stock." The mms designation refers to the characteristic minor protein banding pattern of the chromosome on which *omega* was induced (see Chihara and Kimbrell 1986). "Parental wild type" refers to the $omega^+/omega$, $lcp6^+/lcp6^{mms}$ genotype flies. The remaining offspring follow similar nomenclature except for the fly genotype: $omega/omega$, $lcp6^+/lcp6^{mms}$ which is referred to as "*omega-lcp6* recombinant."

P1: *omega/omega, l3cp6^{mms}/l3cp6^{mms}*   X   *omega$^+$/omega$^+$, l3cp6$^+$/l3cp6$^+$*

F1: *omega$^+$/omega, l3cp6$^+$/l3cp6^{mms}*   X   *omega/omega, l3cp6^{mms}/l3cp6^{mms}*

Back-cross Offspring:

	Parental	Recombinant
Genotype	*omega$^+$/omega, lcp6$^+$/lcp6^{mms}*	*omega$^+$/omega, lcp6^{mms}/l3p6^{mms}*
Phenotype	wild type	wild type
Genotype	*omega/omega, lcp6^{mms}/lcp6^{mms}*	*omega/omega, l3p6$^+$/lcp6^{mms}*
Phenotype	LCP5^{omega}	LCP5^{omega}, LCP6^{omega}

Figure 6: Following the mating scheme presented in figure 5, backcross offspring larvae were dissected and run on PAGE (see Chihara and Kimbrell, 1982). Arrow indicates recombinant LCP6^{omega}. Also shown for comparison are a wild type migration pattern and an *omega* migration pattern.
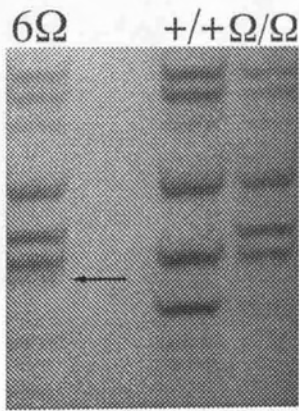
copulation frequency of the corresponding *omega* male cross. For example, from the *omega* male/*omega* female cross (SCF = 0.62) we would expect 0.62 x 171.1to equal the expected number of adult offspring (where 171.1 is the average number of adult offspring from the appropriate control cross, figure 8). 0.62 x 171.1 is equal to 106.08, which is 150% of the observed average number of adult offspring (69.8, figure 8). The other two tested crosses give similar results. Therefore, SCF alone overestimates the number of offspring expected from any cross involving an *omega* male. This gross overestimate supports the proposed synergistic reduction in adult offspring production.

omega matings with heterozygotes demonstrate an increased percentage of females fertilized, an increase in number of adult offspring per female and subsequently, an increased average number of offspring per cross (figure 8, 9, and 10). Both the *omega* male and wild type male crosses, however, demonstrate this hybrid vigor, that is increased fitness through allele recombination of genomes homozygosed through normal laboratory stock maintenance. Note, however, that while the number of offspring in both types of crosses increases, offspring in the omega male cross are still reduced.

Comparing the two mating schemes by which heterozygote offspring are generated, (see figure 8) it is clear that only when omega males are utilized is there a reduction in quantity of adult offspring. This strongly indicates that the diminished offspring numbers are not caused by a developmental abnormality associated with carrying an *omega* allele. That is, animals are not dying after having hatched because they are homo or heterozygote for *omega*.. This suggests that in the other crosses the low fecundity is also due to the *omega* sire. Tracking the numbers of animals at each developmental stage, comparing wild type and *omega* homozygotes would rule out any post egg laying lethality or reduced
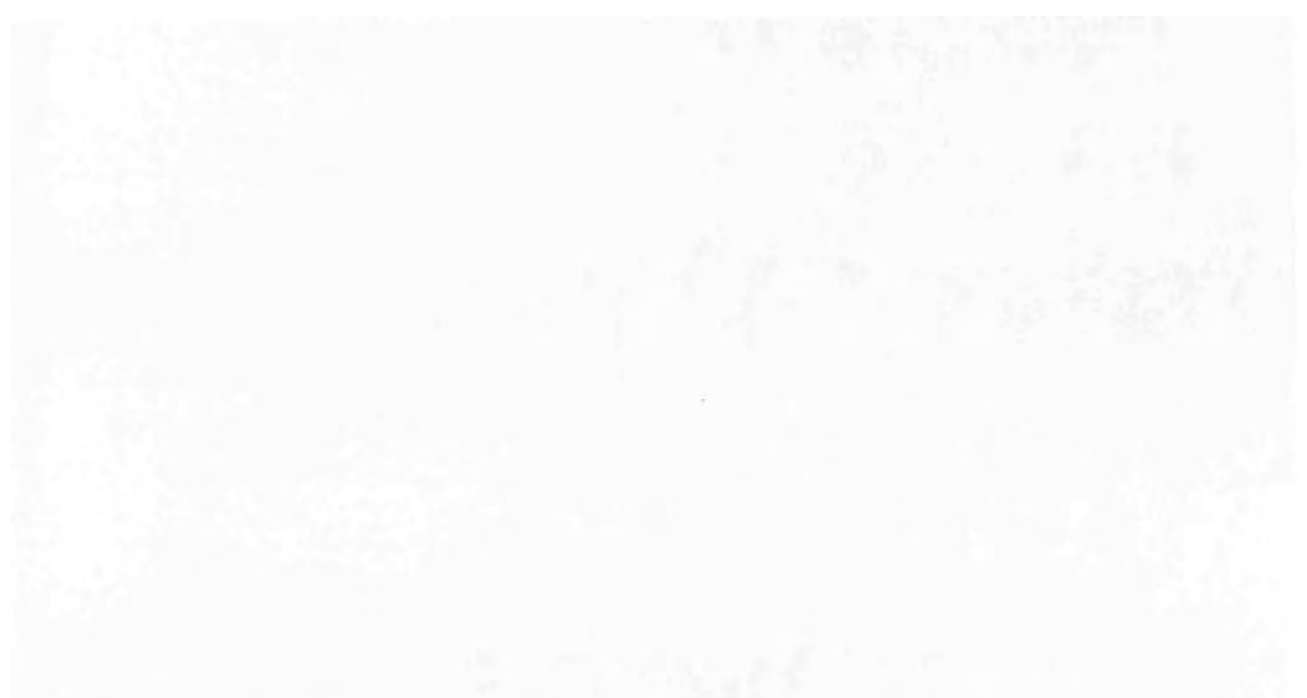
Figure 7: Deficiency Map. Three distinct groups of deficiencies have been isolated in three different laboratories which uncover *omega*: fzM21, Brd6 and the D-5rv series (all shown above the segments in solid lines). Below the segments in dashed lines are four deficiencies which do not uncover *omega*, but using their breakpoints the *omega* locus can be defined as 70E8-71A1. *omega* is shown in a thick solid line. For exact breakpoints of these key deficiencies and other deficiencies tested refer to Table 1. Arrows for D-5rv12 and 14 indicate that the deficiency extends beyond the scope of the presented segments.

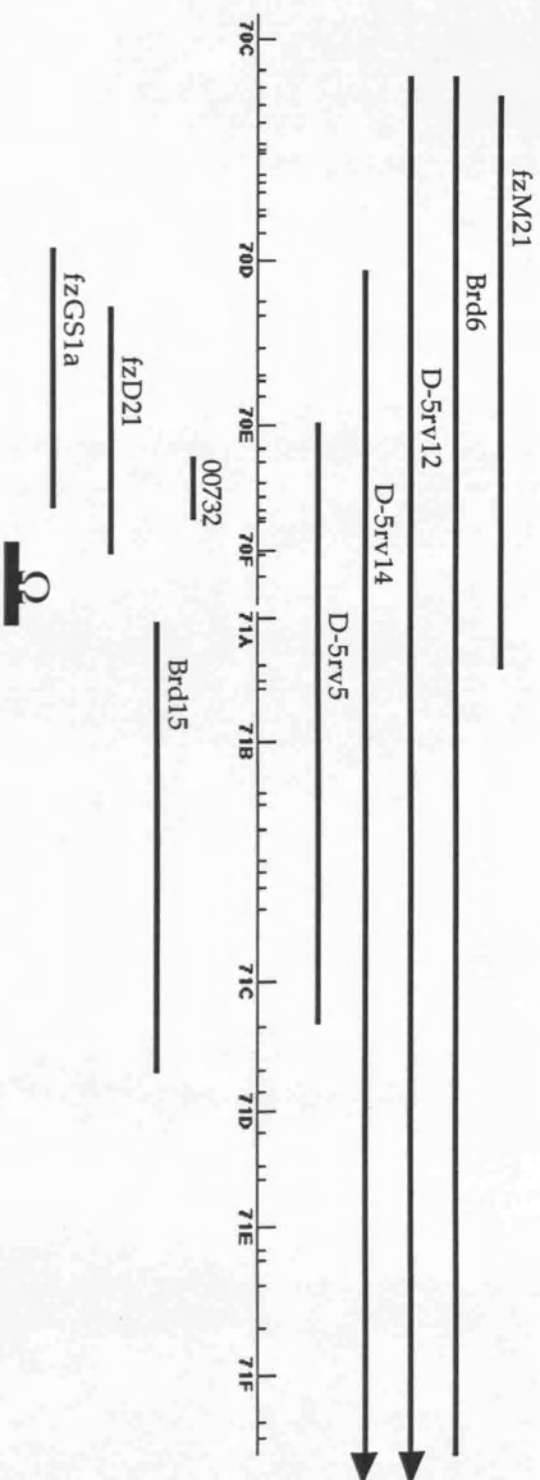Table 1: Past and Current Deficiency Mapping Results. For facility the name and cytology have been provided as well as the date tested and the results. Note that a positive result indicates that the *omega* locus is contained within the breakpoints. Spring/Fall 1995 are replicate trials of the deficiency performed by different members of the lab.

Deficiency	Cytology	Revised Cytology as of 8/4/96	Revised Cytology Source	Result	Date Tested
Df(3L) BK9	68E2-3;69A4-5			Negative	1987
Df(3L)BK140	67F1;68C7-8			Inconclusive[†]	1987
Df(3L)Dm199	Not Found[‡]			Negative	1987
Df(3L)AC1	67A2;67D11-13	67A5;67D9-13	Carpenter, AT	Negative	1987
Df(3L)vin6	68C8-11;69A4-5			Negative	1987
Df(3L)vin7	68C8-11;69B4-5			Negative	1987
Df(3L)vin1	67E55-7;68E3-4			Negative	1987
Df(3L)vin4	68B1-3;68F3-6			Negative	1987
Df(3L)vin3	68C5-6;68E3-4			Negative	1987
Df(3L)fz D21	70D3;70E4-5	70D2;70E8	Carpenter, AT	Negative	Spring 1995
Df(3L)fz GF3b	70B;70C6	70C2;70D5	Carpenter, AT	Negative	Spring 1995
Df(3L)fz GS1a	70C6-15;70E4-6	70D2;70E7	Carpenter, AT	Negative	Spring 1995
Df(3L)fz M21	70E1-2;71C1-2	70E1-2;71D1-2	Spradling	Positive	1990 & Spring/Fall 1995
Df(3L) th 102	72B1;72D1-2			Negative	Spring 1995
Df(3L) Brd 12	70E;71B			Negative	Spring 1995
Df(3L) Brd 15	71A;71C	71A;71C1-71C2	Galewsky,S.	Negative	Spring 1995
Df(3L) Brd 6	70E;71F5	70D-70F;71F	Galewsky,S.	Positive	Spring/Fall 1995
Df(3L) BK10	71C1-2;71F4-5			Negative	1987/Fall 1995
Df(3L)D5R+5	70C;71A2			Negative	Fall 1995
Df(3L)D-5rv12		70C2;72A1	Carpenter, AT	Positive	Summer 1996
Df(3L)D-5rv14		70C2-3;272A	Carpenter, AT	Positive	Summer 1996
Df(3L)D-5rv5		70C3-4;70F5-71A1	Carpenter, AT	Positive	Summer 1996
Df(3L)00732		70E2;70E5	T. Laverty	Negative (6/6)	Summer 1996
Df(3L)st-f13		72C1-2;73A3-4	Flybase (8/4/96)	Negative (6/6)	Summer 1996

[†]The 1987 data shows that only 2 third-instar cuticles were dissected. However the rest of the data suggests that the result would also be negative.

[‡]The cytology for this deficiency has not been found in Lindsley (1992) nor on Flybase.

Figure 8: Average Adult Offspring per Cross. In all cases where *omega* males sired the offspring there is a significant reduction in the number of adult offspring. This figure takes into account both *omega* characteristics which result in reduced adult offspring in crosses with an *omega* homozygote male. For each sample both mean and standard deviation (s) are given.
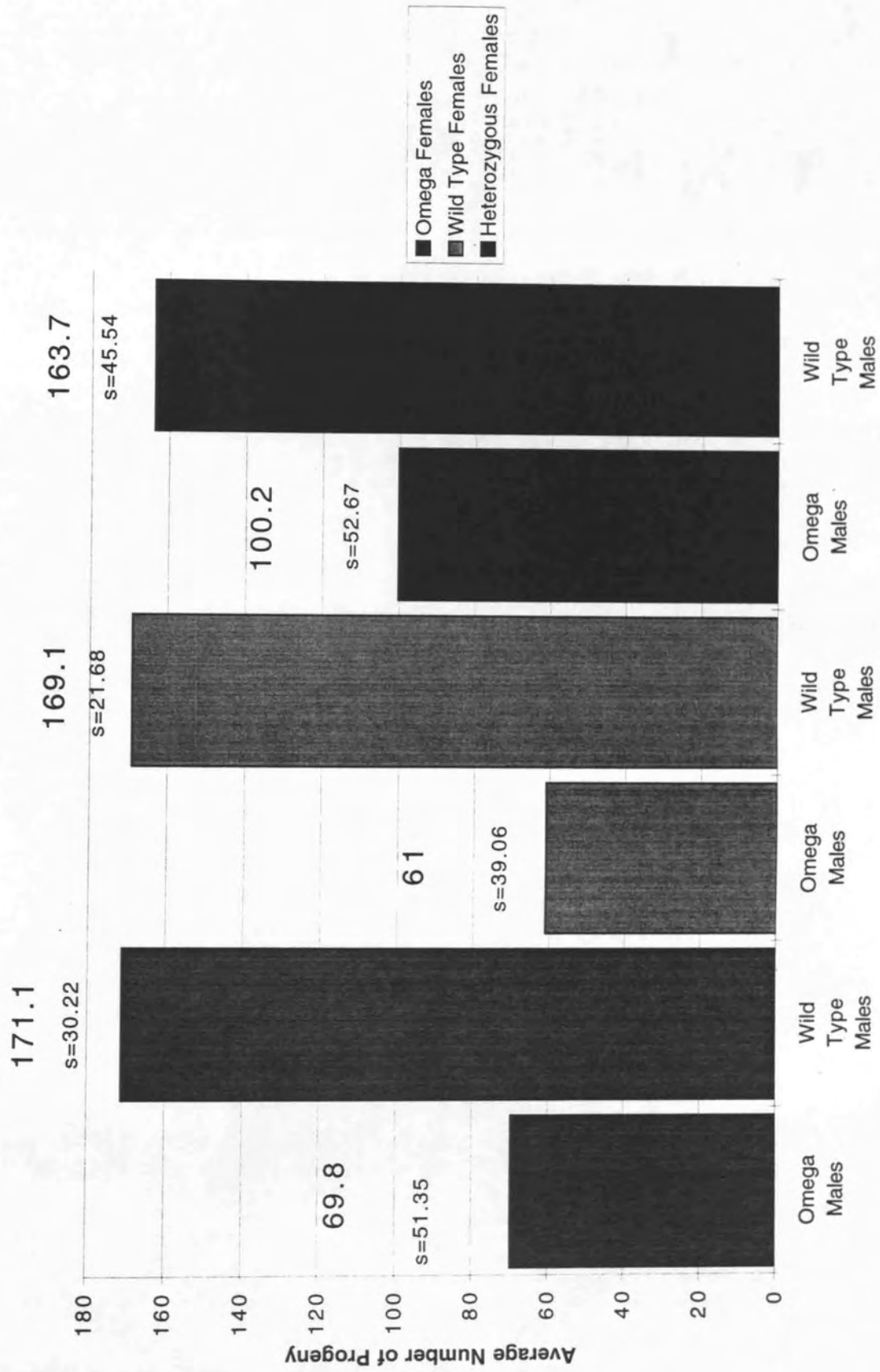
Figure 9: Percent Fertilized Females. For each cross males were introduced to a vial containing five females on day one. On day five the male was discarded and the females were separated into individual vials. Those females which produced offspring post day five were considered fertilized. In all cases there *omega* males are less successful at fertilizing females. 'n' given is the number of females in the sample. n < 50 indicates that some females died/escaped prior to determining whether or not the male had successfully fertilized it.

Average Number of Progeny chart showing:

Legend:
- Omega Females
- Wild Type Females
- Heterozygous Females

Bars (left to right):
- Omega Males: 69.8, s=51.35
- Wild Type Males: 171.1, s=30.22
- Omega Males: 61, s=39.06
- Wild Type Males: 169.1, s=21.68
- Omega Males: 100.2, s=52.67
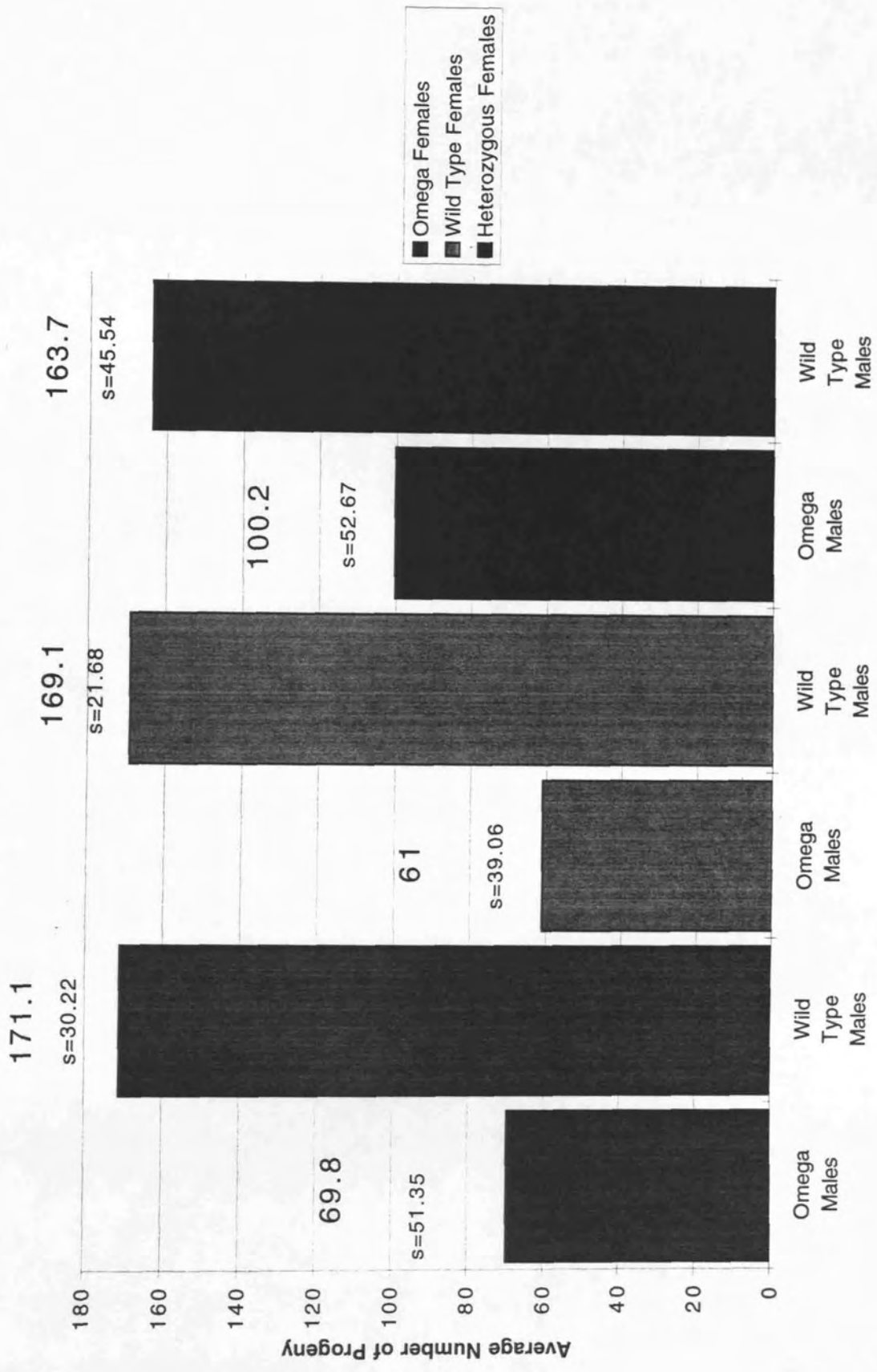- Wild Type Males: 163.7, s=45.54

Figure 10: Average Number of Progeny per Female. Of the females which were successfully fertilized (the value given by 'n') presented here is the average number of adult offspring per female. Note the hybrid vigor illustrated in the heterozygote female crosses. In that case the effects of the *omega* male are still evident when compared to the heterozygote female cross with wild type male. For each sample both mean and standard deviation (s) are given.

Average Number of Progeny per Female

- Omega Females
- Wild Type Females
- Heterozygous Females

131.8 n=33 s=58.93 Wild Type Males

62.3 n=38 s=55.39 Omega Males

65.1 n=45 s=40.08 Wild Type Males

38.7 n=24 s=39.84 Omega Males

84.1 n=49 s=47.61 Wild Type Males

30.4 n=31 s=22.84 Omega Males

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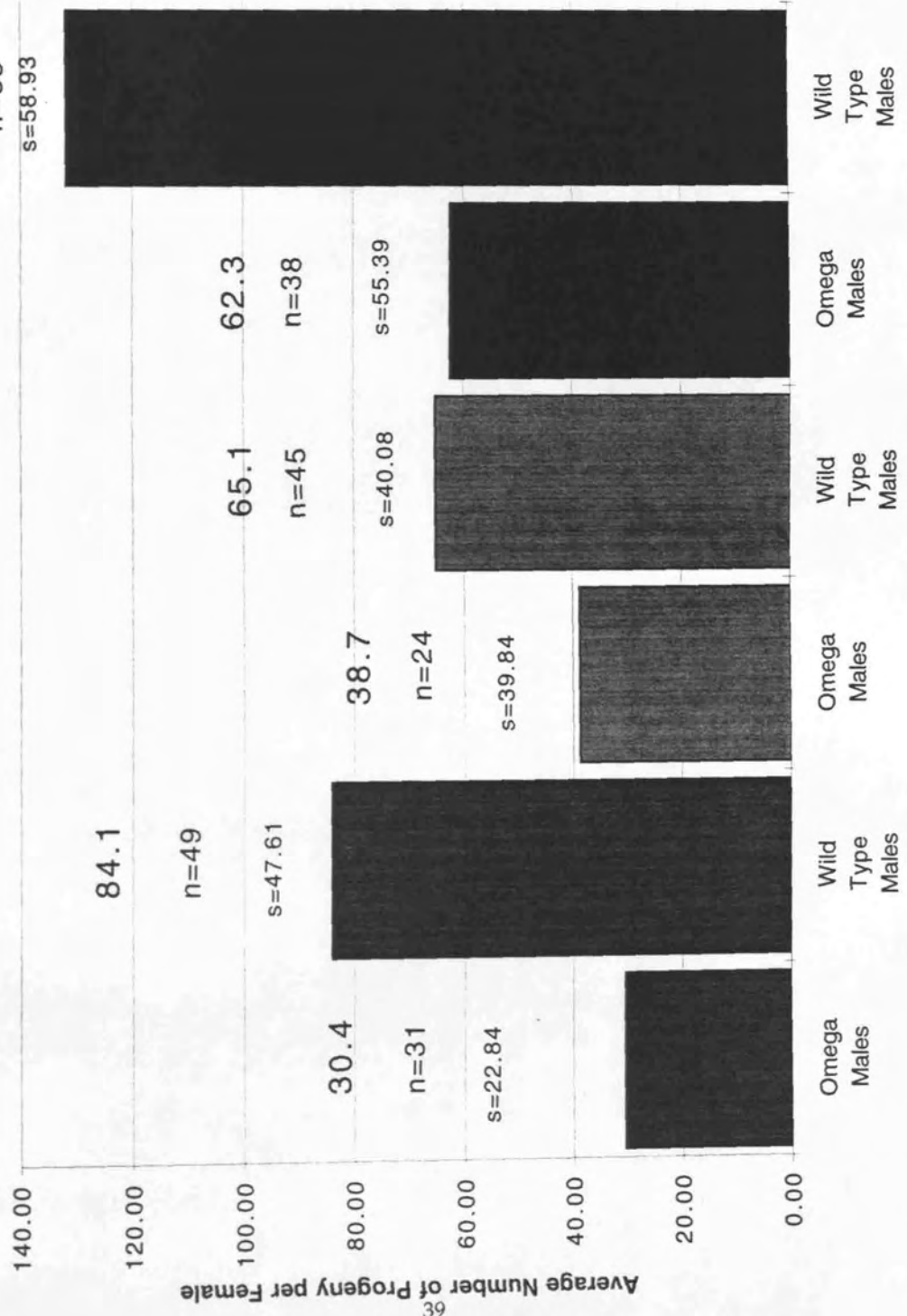
fitness associated with *omega*; taking into account the developmental delay associated with *omega*. Localization of the *omega* locus may be facilitated by a double screening process. The proposed screening process depends on the assumption that the reduced fertility observed in the adult *omega* homozygotes is due to specific omega expression in the testis and upon the deficiency cytology data. First, subtracting a testis specific mRNA preparation with polystyrene bound adult male carcass ss cDNA library. The primary screen would remove all the genes in common, including the house keeping genes, leaving the testis specific mRNA in the flow through. The secondary screen identifies the genes that are common **only** to the testis and the third instar. This screen can easily be done by labeled first strand synthesis cDNA from the testis specific RNA against phage containing third instar cDNA. Depending upon how many clones are finally selected, those that are of most interest (i.e. the longest clones which probably contain larger cDNA fragments) can be identified by an *in situ* hybridization to the 70E5:71A1 region of the third chromosome.

The OMEGA dipeptidylaminopeptidase works on different substrates whose resultant functions vary widely. One the one hand, OMEGA modifies LCP5 whose function in the cuticle is unknown. Cuticle protein null mutants demonstrate that elimination of an individual cuticle protein, itself, is not life threatening, so long as there are sufficient other proteins to replace it. Conversely, *omega* also functions in development and reproduction, two important life processes. The story of *omega* seems to provide a glimpse into the intricacies of life which still elude us. At once this gene is tied by evolution to a mundane task of processing cuticle building blocks, yet simultaneously it comprises a developmental element and is a key modifier in the complex reproductive pathways that make life itself possible for the small fruit fly.

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