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Double Mutant Analysis of Headless and Head Only in the Wasp Nasonia vitripennis.

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INTRODUCTION

Background on *Nasonia vitripennis*

Nasonia vitripennis is a relatively new model organism in the field of developmental genetics. It is a small, solitary, parasitic Hymenopteran found throughout the world. Recent research on *Nasonia* has primarily dealt with its mode of sex determination, and the reproductive isolation of species of *Nasonia* by a parasitic bacteria, *Wolbachia* (Werren 94).

Like many Hymenopterans, *Nasonia* uses the haplo-diploid method of sex determination. In haplo-diploid organisms, fertilized eggs produce diploid females while unfertilized eggs produce haploid males. The system is a bit of a mystery, as it is possible to obtain fertile diploid males by setting triploid females unmated. In some Hymenoptera, sex is determined by the number of different copies of certain genes. Fertilized eggs have produced males after extensive inbreeding. This does not appear to be true in *Nasonia*, however, as much back-crossing has been done without ever producing a single male from a fertilized egg (Bull 1983).

Regardless of the mechanism, haplo-diploidy makes *Nasonia* a particularly useful organism with which to study developmental genetics. In diploid organisms, screening for new mutations is a cumbersome task, requiring two generations to test for mutations. Each female must be mated, her offspring collected, mated to each other, and screened for individuals homozygous for the new mutation. With *Nasonia*, the females can simply be set unmated. The offspring will be haploid males, half of which will be affected by the mutation. Therefore, no back crossing is

required to produce homozygous offspring. Haplo-diploidy also makes stock maintenance easy, as females can readily be tested for mutations before mating. A problem with haploid males arises when working with lethal mutations. Ordinarily, lethal mutations can be carried through the females without much trouble. In constructing a double mutant, however, one must mate a male carrying one mutation to a female carrying the other. This requires the breeding of a diploid male carrying one mutation, for a haploid male can not carry a lethal mutation and survive to mate. Diploid males produce plenty of triploid daughters, but triploid females produce few viable progeny. Only about one in sixteen eggs (in an organism with five chromosomes) gets a full set or sets of chromosomes. Eggs with partial sets suffer serious developmental problems and fail to hatch. The exact procedure used to obtain the double mutant line is outlined in Materials and Methods.

Comparing *Nasonia* to *Drosophila*: common genes

Developmental geneticists have studied the fruit fly, *Drosophila melanogaster* extensively. The development of a fly from fertilization to adulthood is well understood, and the genes involved have been sequenced and mapped. Because *Nasonia* is a promising new model organism, and because it is a few steps removed from the fly evolutionarily, researchers are interested in determining how the wasps developmental processes are similar or different from the fly's. In an effort to identify genes involved in early anterior-posterior determination in *Nasonia*, Pultz, Pitt and Alto (in press) screened mutagenized wasps for mutations affecting embryonic patterning. In these screens, the two mutations in question--*headless* and *head only* were identified. *headless* is necessary for proper formation of the anterior and posterior of the

embryo. A mutation in *headless* results in an embryo lacking head, thorax and last three abdominal segments. *head only* is necessary for proper formation of the entire trunk (thorax and abdomen) of the embryo. When *head only* is missing, the embryo develops a normal head, but lacks most or all of the abdomen, and sometimes part of the thorax as well.

Pultz, Pitt and Alto (in press) compared the defects caused by recessive mutations in *headless* and *head only* to deficiencies caused by mutations in *Drosophila* genes. They found that *headless* mutants appeared similar *hunchback* mutants and *head only* mutants resemble both *nanos* and *caudal* mutants in *Drosophila.* They hypothesized that *hunchback* is homologous to *headless*, because of similarities in the cuticular phenotype (*hunchback* mutants also have incomplete anterior regions). *headless* and *hunchback* mutants also show similar changes in Ultrabithorax and Abdominal A expression, proteins expressed in the abdominal regions in the embryos of wasps and flies. Complete descriptions of the *hunchback* and *headless* phenotypes appear in the results section below. In contrast, *head only* resembles both *nanos* or *caudal.* Pultz Pitt and Alto concluded that, while the *head only* phenotype resembled loss of maternal and zygotic *caudal* more than *nanos* in *Drosophila*, the two organisms are too evolutionarily removed to disregard *nanos* as a possible homolog of *head only* simply based on the phenotypes observed.

hunchback, nanos* and *caudal* in *Drosophila

In *Drosophila*, the mother provides some messenger RNA to the egg before fertilization. At fertilization the RNA is translated into protein which is used by the embryo to establish the initial body plan. This gene contribution is referred to as maternal contribution. The genotype

of the mother is responsible for the maternal effect phenotype of the offspring. Proteins derived from the zygotes own DNA is called zygotic contribution and is dependent on the embryos genotype alone. *hunchback* is an anterior morphogen (a substance involved in body plan formation) provided both maternally and zygotically. Maternal hunchback appears to have no essential function in the fly, and may be an evolutionary vestige. Flies lacking zygotic *hunchback* lack the labial segment (the posterior most head segment), thoracic structures and tail structures, and have fuzed abdominal segments seven and eight. Embryos lacking both maternal and zygotic contribution have a more extreme phenotype, lacking most head segments and having a band of reversed polarity in the anterior of the remaining embryo (Lehmann and Nusslein-Volhard, 1987; Irish, Lehmann and Akam, 1989). When embryos lacking zygotic *hunchback* are stained for Ultrabithorax/Abdominal-A (UBX/ABD-A), an expanded area of expression is visible (White and Lehmann, 1986; Pultz, Pitt and Alto, in press).

nanos is a maternally provided posterior morphogen. At fertilization, *nanos* is translated in the posterior of the egg, where it has been deposited maternally. *nanos* protein represses maternal *hunchback* in the rear of the embryo, by binding to the *hunchback* mRNA and repressing translation (St Johnston, 1993). *nanos*- embryos develop only head, thorax and tail, lacking all abdominal segments. In the absence of maternal *hunchback*, *nanos*- embryos develop normally. This indicates that the only essential function of *nanos* is to repress the transcription of maternal *hunchback*. Even a partial reduction in the amount of maternal *hunchback* mRNA present in the embryo (the progeny of a mother heterozygous for *hunchback*), reduces the severity of the defects. (Irish, Lehmann and Akam, 1989; Pelegri and Lehmann, 1994). *nanos* mutants show a slightly contracted range of UBX/ABD-A expression (Pultz, Pitt and Alto, in

press).

caudal is also a posterior morphogen, and is provided both maternally and zygotically (Macdonald and Struhl, 1986). Loss of maternal or zygotic caudal alone results in an almost normal larvae, as one can partially compensate for the absence of the other. Loss of only maternal *caudal* results in loss of denticle bands on abdominal segments four and eight (not complete loss of segments). Similarly, embryos lacking only zygotic *caudal* lose denticles on segments four and eight, anal tufts and part of the anal pads. Loss of both zygotic and maternal *caudal* causes the embryo to develop with much more severe defects. The *caudal*- phenotype is variable, resulting in loss of even abdominal segments, fusion of some odd segments and loss of all tail structures (Macdonald and Struhl, 1986). The maternal and zygotic mutant phenotype is most similar to the *Nasonia head only* mutant phenotype. Like *nanos* mutants, *caudal*$^{mat\text{-}zyg\text{-}}$ mutants have a slightly contracted zone of UBX/ABD-A expression. However, Caudal and Hunchback proteins have separate roles in patterning the anterior and posterior of the developing embryo.(Pultz, Pitt and Alto, in press).

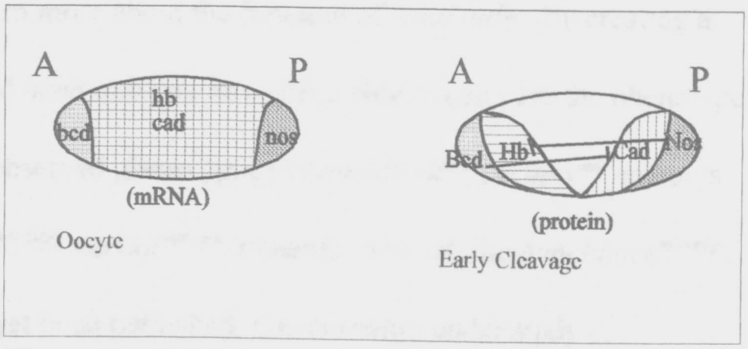

Figure 1: mRNA and Protein gradients in the *Drosophila* embryo.
Before fertilization, *bicoid* and *nanos* mRNA are localized at opposite poles of the egg. *hunchback* and *caudal* mRNA are evenly distributed across the egg. At fertilization, Nanos prevents *hunchback* from being translated in the posterior (P) of the embryo, while Bicoid prevents *caudal* from being translated in the anterior (A) of the embryo, establishing anterior-posterior morphogenic gradients in the embryo.

Why make a double mutant?

Because neither *Drosophila* mutation (*nanos* or *caudal*) is clearly more like *head only* than the other, we felt that more information was necessary to investigate the mode of action of *head only*. How can we learn more about *head only*? By investigating the relationship between *headless* and *head only* and comparing this relationship to that of *hunchback* and *nanos* and *hunchback* and *caudal*, we hoped to learn more about the function of *head only*. By creating a line of wasps carrying both *headless* and *head only*, we have been able to compare the phenotype of a *headless, head only* mutant to the observed phenotypes of $hunchback^{mat--}$, $nanos^{mat-}$ mutants and predicted phenotype of $hunchback^{mat\text{-}zyg-}$, $caudal^{mat\text{-}zyg-}$ mutants. Though the $hunchback^{mat\text{-}zyg-}$, $caudal^{mat\text{-}zyg-}$ mutant phenotype has not yet been published, it is currently under study (C. Desplan, personal communication).

MATERIALS AND METHODS

Origin of stock

All wasp lines were obtained from Dr. MaryAnne Pultz's *Nasonia vitripennis* stocks.

Stock Maintenance and Storage

Stocks were maintained in small glass vials stopped with cotton balls, at room temperature (20-27°C), or 28°C, or 18°C. Pupae were held at the yellow or black stages in the 4°C

refrigerator. Wasps laid eggs on sarcophaga fly pupae grown by Dr. Pultz on site or ordered from Carolina Biological, with the exception of testing unmated females (described below), for which we used Calliphorid pupae. Adult wasps were fed on honey-water until being given fly pupae on which to lay and feed.

Testing females

Because unmated female wasps lay only haploid males, we could easily test females for carried recessive conditions simply by examining their offspring. Before eclosing, females were separated from males. Females were then fed for three days at room temperature after eclosing (five days at 18°C). We then placed each female in a stacked pair of 1ml pipette tips with a single Calliphora pupa (see figure 2). After leaving the female in this apparatus overnight (8-24 hours) at 28°C, we removed and dissected the pupae and placed the eggs on an agar plate to develop further. After approximately an additional 24 hours, the eggs completed their development into hatched or unhatched larvae and could be examined for mutations under a dissecting scope. Under low magnification we recorded ratios of hatched to unhatched eggs and gross body morphology.

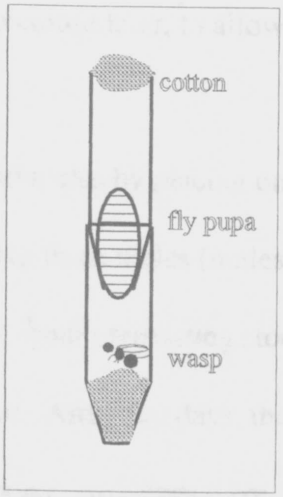

Figure 2: arrangement of testing apparatus

We also mounted the embryos to examine structures not visible under the dissecting scope. We mounted embryos in 90% lactic acid, and observed them briefly on a compound microscope. After a few hours on a heating plate at 56°C, the lactic acid had dissolved the soft tissue allowing us to view the cuticles using darkfield optics.

Producing a line of mothers carrying *head only* and *headless*:

Because *Nasonia* males are naturally haploid, we needed to first create a line of diploid males that were carrying but not expressing one of the mutations of interest. To achieve this, the first necessary cross was between diploid males and *headless*-bearing females. *head only/mickey mouse* females were removed from the refrigerator where they had been stored at yellow pupae stage.[1] They eclosed at room temperature and matured for three days, at which time we tested them as described above. Diploid males were allowed to eclose later, to allow one day of feeding before mating.

Females who laid *head only* progeny in the test were mated to diploid males by placing one female and one male in a small tube. A total of six females were mated to three males (males were used more than once). Diploid males were obtained from Dr Pultz, from preexisting stock. We then set the females in a small glass vial with three sarcophaga pupae. After two days, the tubes were subcultured--pupae were replaced with fresh pupae and the pupae with eggs were placed in the 28°C incubator to develop. Tubes were then subcultured twice each week. The egg-bearing pupae incubated for seven days, or until the wasps reached the yellow-pupae stage. At this time, we broke open the fly pupae, sorted the wasps by sex, left some females at room temperature to eclose and placed excess stocks in the refrigerator as backups. The following generations proceeded according to the table below, each generation requiring at least 15 days to complete.

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[1] *mickey mouse (mm-)* is a marker closely linked to head only. The affected individuals (mutant for the *mm* gene) have bulgy eyes. *st318-*, scarlet eye color, is also closely linked to *head only*. *reverent (rev-)* is linked to headless, and results in turned in legs on effected pupae.

Table 1: Generations required for production of double mutant. Individuals in bold are the direct progeny of the previous generation, all others are from stocks. Only desired genotypes are listed.

Gen.	Mothers	Fathers (if any)	Number of females set
1	*ho/mm*	diploid	6
2	**triploid (*ho*/+/+)**	set unmated	20
3	*hl/rev*	**diploid *ho*/+**	10
4	**triploid (*hl/ho*/+)** tested line 3 and 4 looked good for *hl*. Attempted to get diploid females carrying both mutations, but not successful due to insufficient numbers.	set unmated to get diploid males, below	30
5	*ho-, mm+,st318-* *ho+,mm-, st318-*	**diploid (*hl*/+)**	5
6	**triploid *ho-,st318-/ho+,st+/ho+,st+ hl-/hl+/hl+*** (discarded all cultures with mm sons)	***st318- mm-* (brothers)**	35
7 Yay!!	**diploid *ho-, st318-; hl-*** ***ho+, st318-; hl+***	*comp+* (a wild type line)	3
8	***ho-, st318- ; hl-*** ***ho+, st318+; hl+***	*st318-*	8

Analysis of Double Mutant Phenotype

Once we obtained the double mutant line, we set females unmated to collect male embryos for observation. As expected, each female carrying both mutations laid approximately 25% wild type, 25% *headless*, 25% *head only* and 25% double mutant offspring (table 2). To view the cuticle phenotype, we collected embryos and larvae as described in testing, above, mounting on slides in lactic acid and allowed to clear. Slides were analyzed and photographed using darkfield illumination.

We also collected younger embryos and stained for Ultrabithorax/Abdominal A and Engrailed. We conducted two collections, one at 1 to 4 hours after gastrulation, and another 9 to 12 hours after gastrulation. Embryos were collected on agar plates after a three hour lay and then the plates were incubated for 11 or 19 hours at 28°C before embryos were fixed.

Table 2: Number of each class of embryos on slides used for double mutant analyses.

	wild type	*headless*	*head only*	double mutant
cuticle prep	15	17	13	12
UBX/ABD-A stain	7	5	5	6

Fixing Embryos

We fixed and stained embryos using a modified version of the protocol described in Goldstein and Fyrberg (1994).

1) Embryos were transferred from the plate to a five ml plastic tube and shaken vigorously in a 1:1 solution of heptane and 4% paraformaldehyde in 1X PBS for 10 minutes, to fix the embryos. We then removed as much as possible of the heptane and paraformaldehyde, taking care not to disturb the embryos at the interface. A modified pasture pipette, with a drawn out tip aided in this step, by decreasing the number of embryos sucked up and stuck to pipette while removing phases.

2) A -80°C 1:1 mixture of heptane and methanol was added to the tube, shaken for a few seconds then shaken under lukewarm tap water to warm the embryos quickly. As tube warmed, we reduced the water temperature to room temperature. We continued to shake the tube for 2-5 minutes. This step caused the vitelline membranes to pop off the embryos. The intact embryos that lost their membranes sank, while vitelline membranes remained at the surface of the tube, or at the heptane-methanol interface. The upper part of the solution could be removed, leaving the embryos at the base of the tube. Embryos were then transferred to a glass tube for counting and observation. Care was taken to cool the embryos quickly, warm them quickly without overheating them, and shake moderately hard but not quite vigorously.

3) Embryos were rinsed several times in methanol and transferred to a 500μl tube before being stored at 4°C. (Embryos thus fixed may be stored for up to two weeks before staining.)

Antibody application

The procedure was carried out in a 500 microliter eppendorf tube, all incubations at room temperature and on a rocker unless otherwise indicated.

UBX/ABD-A

1) After adding one part hydrogen peroxide per 100 parts methanol, the embryos were incubated for 15 minutes. Washing in hydrogen peroxide prevents endogenous peroxidases from causing large amounts of background staining in the embryo.

2) Embryos were rinsed in methanol three times to get rid of hydrogen peroxide, methanol was removed, and embryos were rinsed twice in PBT (5 minutes each) to rehydrate, then incubated for an additional 30 minutes. (PBT: 0.1%bovine serum albumen, 0.1% Triton, in 1x Phosphate-Buffered Saline)

3) Goat serum was added to 5% of total volume and tube was incubated for an additional 30 minutes, to flood the embryo with protein and reduce non-specific binding of the antibodies.

4) The primary antibody (mouse anti-UBX/ABD-A FP 6.87) was added to a concentration of 1:7 with the PBT and goat serum mixture, and the tube was incubated overnight at 4°C.

5) 14 hours later, the embryos were washed in PBT-- three times for five minutes, and five times for 20 minutes.

6) Goat serum was again added to 5% of total volume, and incubated for thirty minutes.

7) Secondary antibody was added to a total concentration of 1:250. We used a horseradish-peroxidase conjugated goat anti-mouse IgG antibody. Mixture was incubated for two hours then washed as for primary antibody (step 5, above). The younger group of embryos were stained

first, so the older embryos incubated in PBT for an additional 30 minutes.

DAB black (Nickel) reaction

8) After the wash, we removed the supernatant (PBT) and added 3μl 8% $NiCl_2$ and approximately 400μl DAB solution to the reaction tube (0.3mg/ml DAB diluted 1:1 in PBS 0.1% TWEEN). Embryos were then incubated for at least two minutes.

9) Hydrogen peroxide was added to $1.2x10^{-3}$% and the reaction was inverted to mix and carefully observed for color change. We put a few embryos in a dish and observed stain developing under a dissection scope.

10) When the background color appeared to be developing faster than the stain (after about 5 minutes), we rinsed off the DAB by diluting in PBT, and washing twice for 30 minutes each..

The presence of Nickel in the peroxidase reaction results in a black stain.

Engrailed

Approximately half of each collection of embryos stained for UBX/ABD-A were retained as backup. We stained the other half with an antibody against Engrailed in an effort to clarify segment boundaries, and make analysis of the UBX/ABD-A stain easier.

11) Embryos were incubated in goat serum, as in step 3 above, then primary antibody (mouse-anti Engrailed, EN 4D9) was added at a 1:1 concentration, and embryos incubated overnight at 4°C (about 17 hours). Washings proceeded as in steps 5 and 6 above. In the final wash, the

early-collection embryos were allowed to wash for an additional 30 minutes before adding goat serum, staggering them to avoid the additional 30 minutes of incubation before staining (as in step 7, above).

12) Secondary antibody (HRP conjugated goat anti-mouse) was added to the tube, to and incubated for 2 hours before being washed as in step 5.

DAB orange reaction

Orange reaction proceeded like black reaction, but no nickel chloride was added to the reaction. In the absence of nickel the peroxidase reaction results in an orange-brown product:

Mounting on slides

1) Embryos were rinsed twice in PBS, and twice, for five minutes each in 50%, 70%, 90% and 100% ethanol. Excess ethanol was wicked away and methyl salicylate added to fill tube. Embryos were held at 4°C until mounting.

2) Embryos were mounted on a glass slide with coverslip in a few drops of methyl salicylate, and observed and photographed using light microscopy and Differential Image Contrast microscopy.

RESULTS AND DISCUSSION

Cuticle Prep

The wild type *Nasonia* larva has 13 denticle belts (three thoracic and ten abdominal), head

with mouthparts and antennal papillae, and tube-like anus . Segments can be identified by the presence of spiracles on the second thoracic segment, and the first three abdominal segments (figure 3A).

In the *headless* embryo, the head and thoracic segments are deleted, as well as the last three abdominal segments (eight through ten). Sometimes disorganized thoracic denticle belts are present on the dorsal surface only, but complete segments are not formed. As shown in figure 3B, this results in an embryo that has no head structures and a truncated tail, without the anal tube. The protrusion at the anterior of the embryo is the epistoma, the only remaining head structure. The *headless* mutant does not hatch.

In the *head only* embryo, most of the abdomen and sometimes even the thorax is deleted (figure 3C and D). Like the *headless* embryo, the *head only* embryo can not hatch. The *head only* phenotype is variable, depending on temperature and on the genetic background of the mutant. The head of the embryo is always formed completely, but the posterior of the embryo may be missing anywhere from seven to twelve segments. Often *head only* embryos lack a tail altogether, and simply end in an open hole after the first denticle belt or two. When the tail is intact, however, it is pointed like the wild-type's, but lacking complete anal structures.

The double mutants appear almost barrel-like in their cuticular phenotype (figure 3E and F). The embryos have two to four denticle belts, but little or no visible segment boundaries. They have a protrusion at the anterior, resembling the epistoma on *headless* embryos, and a rounded posterior. Though it is impossible to identify individual segments on the double mutant, the posterior is clearly missing more segments than the posterior of a *headless* mutant, which only loses the last three segments and the tail. The phenotype of the double mutant has clear aspects

of both *headless* and *head only* defects, resulting in the loss of head and tail as well as regions of the thorax and abdomen.

The cuticular phenotype of the *headless, head only* double mutant indicates that *headless* and *head only* may have separate functions in the anterior and posterior. Because *hunchback* and *caudal* also have separate roles in patterning the anterior and posterior of the developing fly embryo, this combined phenotype suggests that the relationship between *headless* and *head only* may be more like the relationship between *hunchback* and *caudal* than like the relationship between *hunchback* and *nanos*.

Ultrabithorax expression

In order to further investigate the relationship between *headless* and *head only*, we stained embryos for the proteins Ultrabithorax and Abdominal A (UBX/ABD-A). We compared the expression of UBX/ABD-A in the single *headless* or *head only* mutants to the double mutant in both early (1-3 hours after gastrulation) and late (8-11 hours after gastrulation) embryos. We also stained for Engrailed to help identify segments in the mutant embryos. In the late stage embryos, expression of the proteins was restricted primarily to the nerve chord, making patterns of expression difficult to observe. We emphasized the younger embryos in our analysis of UBX/ABD-A expression, as they were more instructive. In the early embryos UBX/ABD-A expression was in the initiation stages, where it is under control of the gap genes.

UBX/ABD-A is expressed in the wild type *Nasonia* embryo weakly between the second and third thoracic segments, and strongly through the first seven abdominal segments (figure 4A). Engrailed is expressed in the posterior of each segment of the developing embryo, including the

antennal, intercalary, mandibular, maxillary and labial segments, and twelve trunk segments (Pultz, Pitt and Alto, in press).

The region of UBX/ABD-A expression in the *headless Nasonia* embryo is greatly expanded (figure 4B), extending from what would be the head of a wild type embryo, to just short of the posterior end. The UBX/ABD-A band overlaps Engrailed expression in most of the embryo, making the Engrailed stripes obscured on the stained embryo. In the *head only* embryo, UBX/ABD-A expression is contracted to a narrow band in the abdomen. Engrailed expression is visible in the head and thorax of the embryo (figure 4C).

A fourth class of embryos was found in the UBX/ABD-A, Engrailed staining. Because approximately one quarter of the embryos fell into this class, they were assumed to be the double mutants. The expression of UBX/ABD-A in these embryos is especially interesting. The UBX/ABD-A expression begins just behind to the anterior tip of the embryo, and ends just before the posterior tip, similar to the expression in *headless* mutants. However, unlike *headless* mutants, there is a gap in UBX/ABD-A expression at the back of the anterior half of the embryo. Engrailed stripes do not clarify the exact location of this gap, but it appears to be in the area of the last thoracic segments and the first abdominal segments (figure 4D).

The UBX/ABD-A stained embryos present an interesting dilemma. Because *headless* and *head only* mutations have opposite effects on UBX/ABD-A expression, we considered the possibility that one phenotype might be epistatic to the other in the double mutant. If we assume that the role of *headless* is to repress UBX/ABD-A expression in the anterior of the embryo, we can formulate two models for the role of *head only* that lead to an epistatic effect in the double mutant. If the only role of *head only* is to repress headless in the posterior of the embryo, the

loss of both *headless* and *head only* would result in a *headless* phenotype, because without *headless*, *head only* would not be needed. If the role of *head only* is to activate UBX/ABD-A in the posterior, the phenotype of the double mutant would resemble the *head only* phenotype, because without *head only* to activate UBX/ABD-A, there is nothing for *headless* to repress. The gap in the band of UBX/ABD-A expression in our double mutant indicates that neither of these interactions is true.. Therefore, there may be other gene interactions taking place.

We have developed a model to explain the gap in UBX/ABD-A expression in the *headless, head only* double mutant (figure 5). If a gene *c* represses UBX/ABD-A expression in the region in question (region x), but is repressed by *head only,* the lack of *headless* would still cause expansion of UBX/ABD-A, but without *head only* to repress *c* in region x, *c* would create the gap.

Further study

Note that the function of *hunchback* and *caudal* have both maternal and zygotic components in *Drosophila*, while *nanos* acts purely maternally. We have only identified zygotic phenotypes of the *Nasonia* genes *headless* and *head only.* This does not preclude the existence of maternal contribution of *headless* and *head only*. We are investigating this possibility by comparing the phenotype of single mutant offspring from mothers carrying both mutations, to single mutant offspring of mothers carrying only one mutation.

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FIGURES

Figure 3: Darkfield images of embryos. A) Wild type larvae. Note the complete mouth structures. First thoracic (T1) and first abdominal (A1) segments labeled. B) *headless*- embryo. Arrow indicates epistoma. C) and D) *head only*- embryos. Note variability in phenotype. E) and F) *headless, head only* double mutant. Arrow indicates epistoma.

Figure 4: Embryos stained against Engrailed (Eng) and Ultrabithorax/Abdominal-A (UBX/ABD-A) A) Wild type embryo. B) *headless* mutant embryo. C) *head only* mutant embryo. D) *headless, head only* mutant embryo. Arrows indicate range of UBX/ABD-A expression and Eng stripes, as labeled.

Figure 3: Darkfield images of embryos.

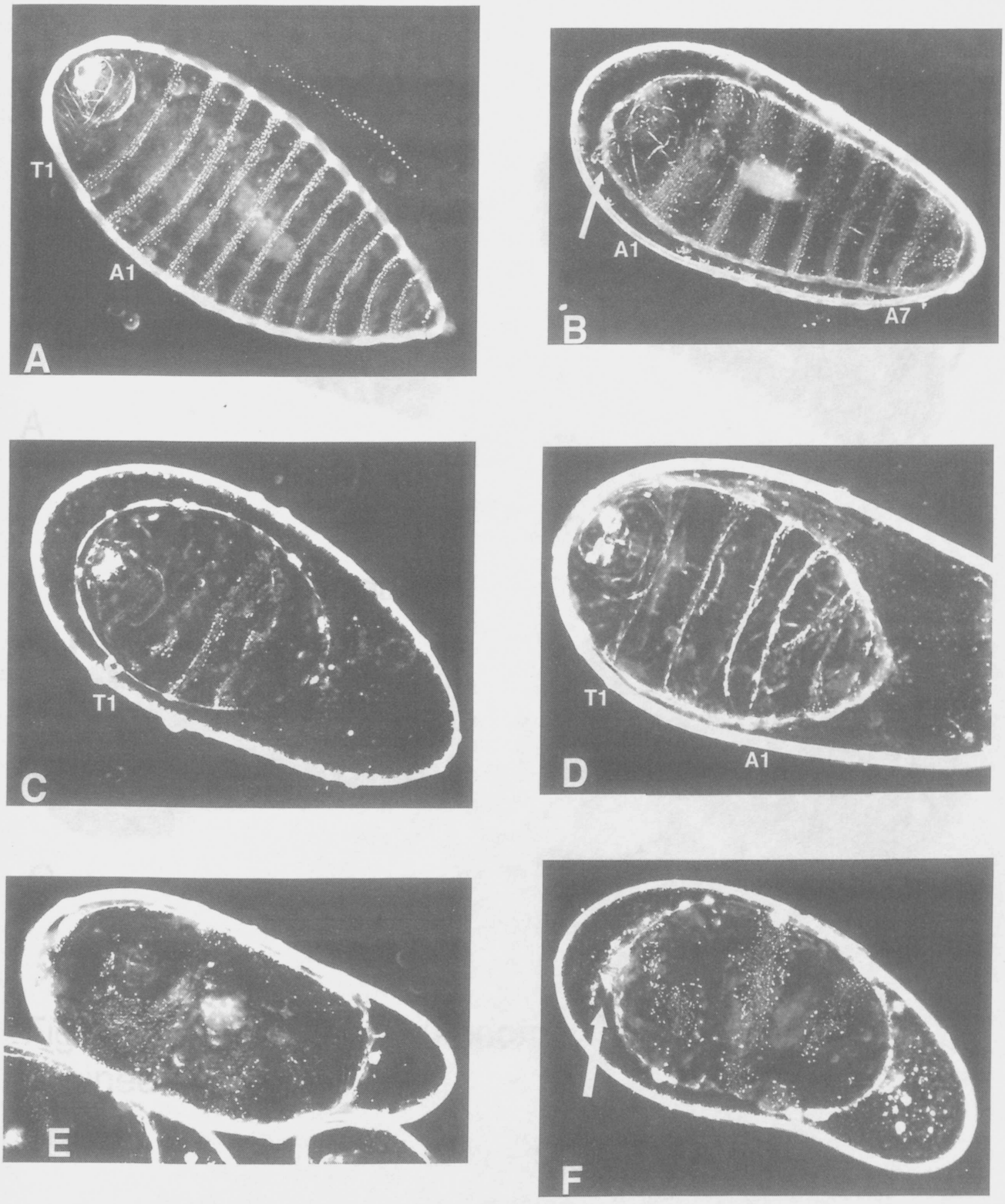

Figure 3: Darkfield images of embryos.

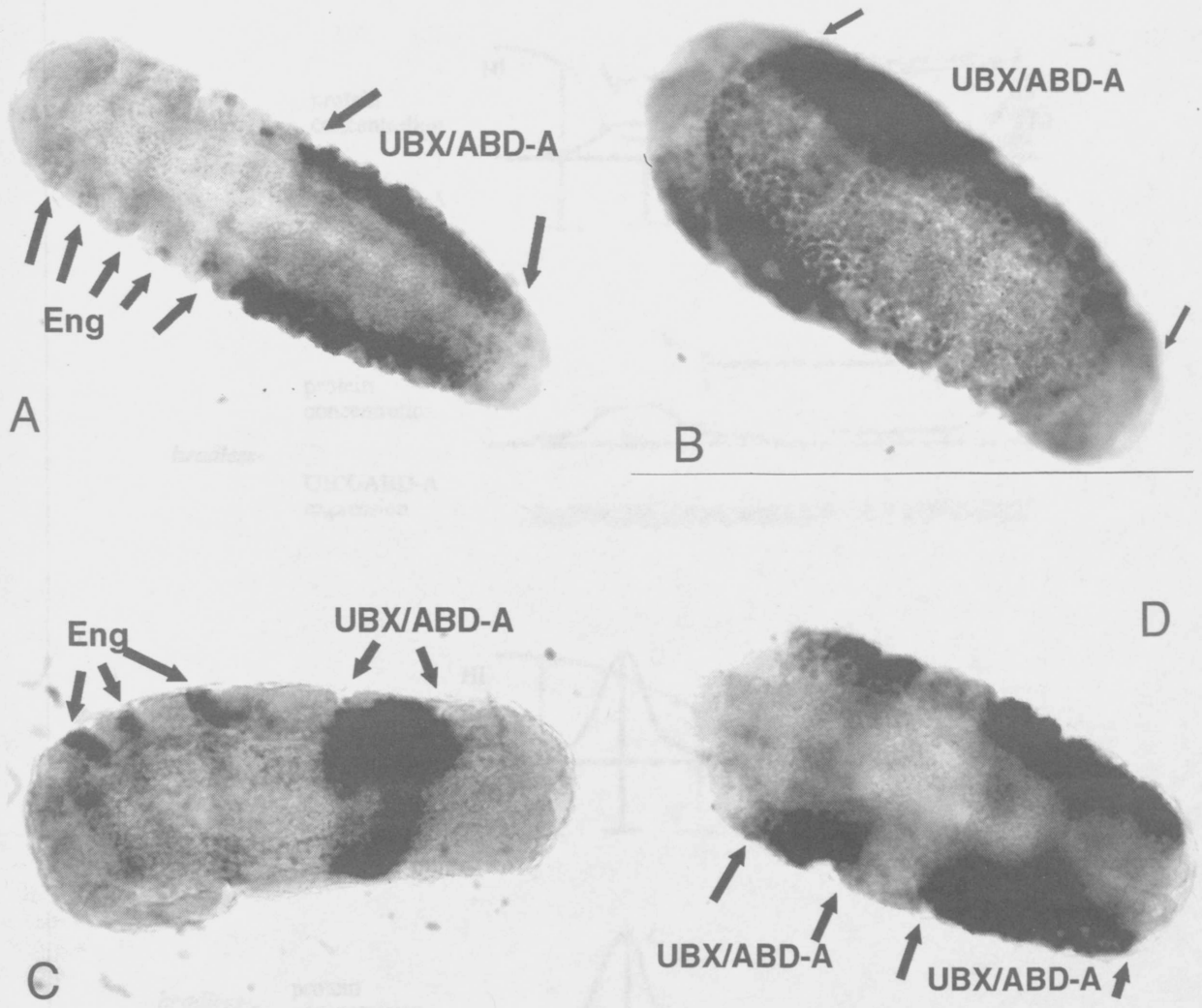

Figure 4: Ultrabithorax/Abdominal-A and Engrailed stained embryos.

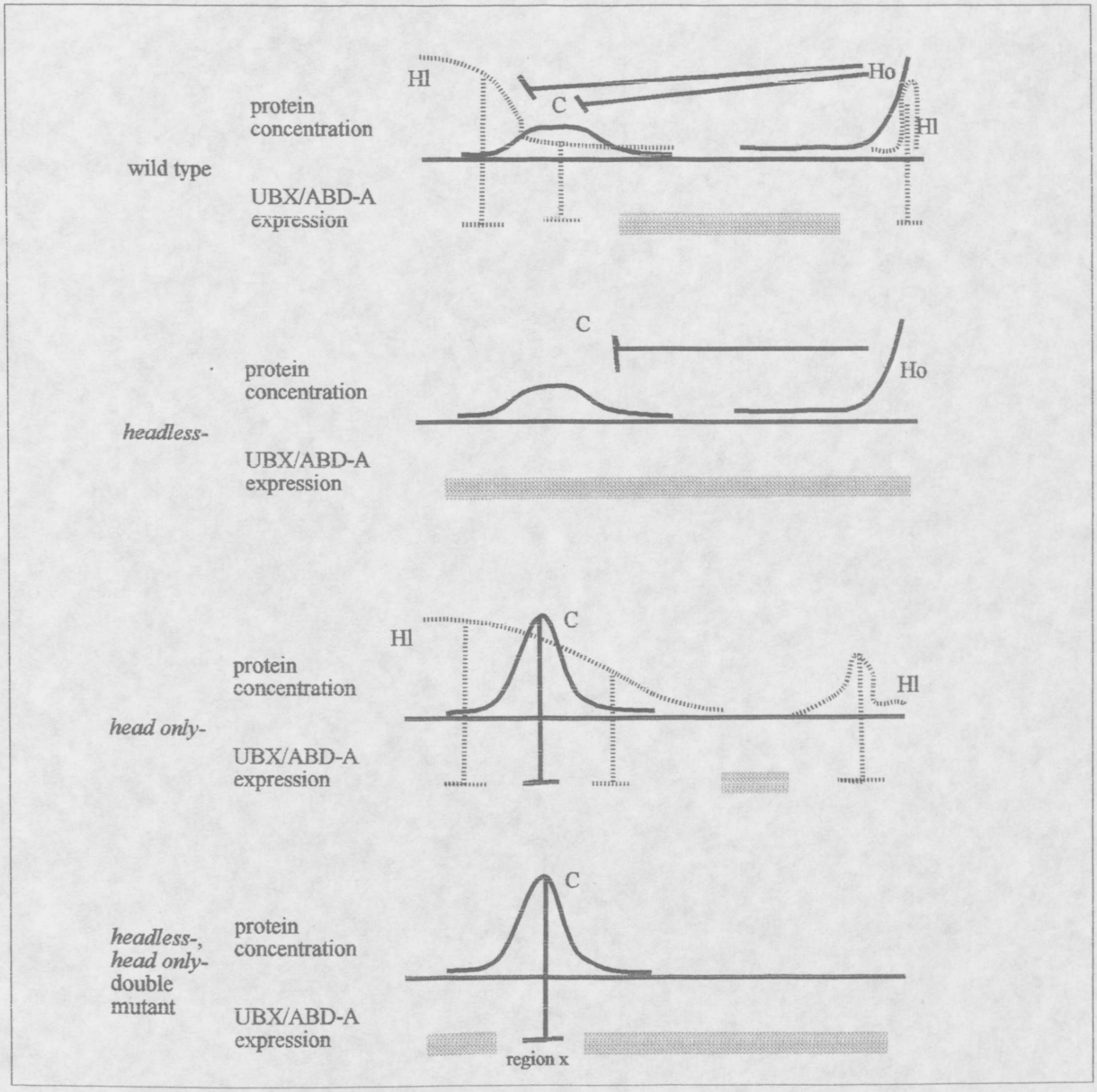

Figure 5: Simplest model of *headless*, *head only* and *c* control of UBX/ABD-A expression.
In the wild type embryo, Headless (Hl) represses UBX/ABD-A in the anterior and posterior of the embryo. C is repressed by Head only (Ho) in the thorax, and Hl is repressed by Ho in the abdomen (excluding the posterior-most tip).
In the *headless*- embryo, the domain of UBX/ABD-A is expanded in the anterior and posterior because Hl is not present to repress it. C is still repressed by Ho.
In the *head only*- embryo, Ho is not present to repress Hl, so Hl represses UBX/ABD-A in a larger area. C is also free to repress UBX/ABD-A.
In the double mutant, the domain of UBX/ABD-A is expanded as in the *headless* mutant, but C is not repressed by Ho, so C represses UBX/ABD-A in region x.