

# Silencing of an abdominal *Hox* gene during early development is correlated with limb development in a crustacean trunk

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**SUMMARY** We tested whether *Artemia abd-A* could repress limbs in *Drosophila* embryos, and found that although *abd-A* transcripts were produced, ABD-A protein was not. Similarly, developing *Artemia* epidermal cells showed expression of *abd-A* transcripts without accumulation of ABD-A protein. This finding in *Artemia* reveals a new variation in *Hox* gene function that is associated with morphological evolution. In this case, a HOX protein expression pattern is

completely absent during early development, although the HOX protein is expressed at later stages in the central nervous system in a “homeotic-like” pattern. The combination of an absence of ABD-A protein expression in the *Artemia* limb primordia and the weak repressive function of *Artemia* UBX protein on the limb-promoting gene *Dll* are likely to be two reasons why homonomous limbs develop throughout the entire *Artemia* trunk.

## INTRODUCTION

*Hox* genes are transcription factors that specify regional identities along the anterior–posterior axis of both invertebrates and vertebrate embryos. In many metazoans, *Hox* genes are found within one or multiple clusters and the order of *Hox* genes on the chromosome typically correlates with the order of their anterior expression boundaries on the anterior–posterior axis (Lemons and McGinnis 2006). In arthropods, evolutionary variations in the expression patterns and functions of *Hox* genes have been intensively studied (Carroll et al. 2005). This is because *Hox* genes were initially discovered in an arthropod model organism, *Drosophila melanogaster*, and because of the enormous morphological diversity in arthropods, particularly in appendage morphology. Among the Pancrustacea (crustaceans+insects—Regier et al. 2005), the hexapod insects are at an extreme in terms of appendage modification, having lost limbs in the posterior trunk and forming limbs on only three trunk segments. At another extreme are the branchiopod crustaceans with homonomous trunk segments, all bearing limbs, a state that is believed to resemble the trunk morphology of the common ancestor of insects and crustaceans (Regier et al. 2005; Vanhook and Patel 2008; Budd and Telford 2009). It has been proposed that the extreme modification of limb morphology seen in the insect lineage was accomplished in part by evolutionary changes in the expression patterns and functions of the pos-

terior trunk *Hox* genes *Ultrabithorax* (*Ubx*) and *abdominal-A* (*abd-A*) (reviewed in Hughes and Kaufman 2002a, b; Carroll et al. 2005).

Most studies on the role *Hox* genes have played in evolving arthropod appendage morphology have involved analyses of *Hox* gene expression patterns in different species (Hughes and Kaufman 2002a, b; Angelini and Kaufman 2005). For example, in a few different crustacean species, the anterior expression boundary of the HOX protein UBX correlates with the transition between segments that bear maxillipeds—feeding appendages, and those that bear limbs—locomotory appendages (Averof and Patel 1997; Shiga et al. 2006). In the crustacean *Porcellio scaber*, maxilliped development in the first thoracic segment (T1) is associated with the expression of the HOX protein SCR protein in the developing T1 appendage (Abzhanov and Kaufman 1999). A variety of evidence of this type suggests variations in *Hox* expression patterns have modified appendage morphology during arthropod evolution, just as variations of *Hox* expression patterns can modify appendage morphology during *Drosophila* development (Struhl 1982; McGinnis and Krumlauf 1992).

A few studies have found evidence that HOX protein evolution has contributed to limb repression and diversity (Galant and Carroll 2002; Ronshaugen et al. 2002; Shiga et al. 2002). For example, when tested in *Drosophila* embryos, the UBX proteins of insects were found to have a stronger repressive function on the appendage-promoting gene *Distal-*

less (*Dll*) than UBX proteins from a branchiopod crustacean (*Artemia franciscana*) or an onychophoran (*Akanthokara kaptensis* sp.). This finding correlates with the observation that *Artemia* and *Akanthokara* develop limbs in body segments that express UBX protein. For *Artemia* UBX, it was suggested that a consensus casein kinase 2 (CK2) site (Pinna 1990) in the C-terminal region might inhibit UBX limb repressive function (Ronshaugen et al. 2002), and one goal of the current study is to test this hypothesis.

We also wished to gain additional insight into evolutionary diversity in HOX and body plan variation by studying the function of the *A. franciscana* *Hox* gene *abd-A*. It has long been proposed that the cluster of *Hox* genes arose from successive tandem duplications (Lewis 1978) and it is believed that the duplication which generated *Ubx* and *abd-A* occurred before the evolution of a common ancestor of crustaceans and insects (Averof and Akam 1995). In a wide variety of insects, genetic evidence and in situ analysis of expression patterns indicate that *abd-A* completely represses *Dll* and limbs (Sánchez-Herrero et al. 1985; Tear et al. 1990; Nagy et al. 1991; Vachon et al. 1992; Sánchez-Herrero et al. 1994; Shippey et al. 1998; Lewis et al. 2000; Angelini et al. 2005; Zhang et al. 2005). In the crustaceans *P. scaber* (woodlouse) and *Procambarus clarkii* (crayfish) the early phase of *abd-A* expression is correlated with trunk segments that develop small, highly modified appendages, consistent with the model that ABD-A partially represses limb development in at least some crustaceans (Abzhanov and Kaufman 2000a, b; Angelini and Kaufman 2005). However, the crustacean *Artemia* develops homonomous limbs on all of its trunk segments, even though previous studies have reported that UBX and ABD-A proteins are expressed throughout the developing trunk region (Averof and Akam 1995; Shiga et al. 2006). Based on this, it seemed possible that the *Artemia* ABD-A protein (like *Artemia* UBX) might have a reduced repressive function on *Dll* and allow the development of limbs.

To test the function of *Artemia abd-A* on limb development, we induced the expression of an *Artemia abd-A* coding sequence in *Drosophila* embryos. This crustacean version of full-length *abd-A* had no effect on modifying limb development, but for a completely unexpected reason. *Artemia abd-A* transcripts were produced in *Drosophila* embryos, but ABD-A protein was not. This result was paralleled when we analyzed the expression pattern of ABD-A protein in developing *Artemia*. During the early larval stages when *Artemia* limb primordia are forming, *abd-A* transcripts are detected at low levels, but no ABD-A protein is detected in larval trunk segments. This finding in *Artemia* reveals yet another variation in *Hox* gene function that is associated with morphological evolution. In this case, a HOX protein expression pattern is completely absent in the epidermis during early development, although the *Hox* protein is expressed at later stages in the central nervous system in a “homeotic-like” pattern. The

combination of an absence of ABD-A protein expression in the *Artemia* limb primordia and the relatively weak repressive function of *Artemia* UBX protein on *Dll* are likely to be two reasons why homonomous limbs develop throughout the entire *Artemia* trunk.

## MATERIALS AND METHODS

### In vitro CK2 assays

N-terminal GST-fusion proteins of interest were expressed in *Escherichia coli* and purified. In vitro CK2 kinase reactions were performed using 65 ng/μl protein, 3 U/μl CK2 (NEB, Ipswich, MA, USA, catalog #P6010S), 3 nmol ATP, and 5 μCi α-<sup>32</sup>P ATP. All of the reaction was loaded onto a 10% SDS-PAGE gel then exposed to film at −80°C. The acrylamide gel and autoradiograph were scanned and relative amounts of protein and phosphorylation were determined using ImageJ. The level of *Artemia* UBX phosphorylation was set at 100% and phosphorylation levels for the other proteins was calculated relative to *Artemia* UBX, after normalizing for the amount of protein. The percentages are an average of two separate experiments.

### Simultaneous protein detection and FISH

*armadillo*-Gal4 (*arm-GAL4*) virgin females were crossed to males containing the appropriate UAS-UBX construct. Embryo collection was performed as described in Kosman et al. (2004) with the following modifications. Washes were carried out for 5 min in 1 ml volumes, unless otherwise noted. Embryos were rocked in 1:1 xylene:ethanol mixture for 30 min. Embryos were washed twice in 100% ethanol, then twice in 100% methanol. Embryos were rehydrated in a graded methanol:H<sub>2</sub>O series (75%, 50%, 25%), then in 100% H<sub>2</sub>O. Embryos were then permeabilized with 80% acetone in H<sub>2</sub>O for 10 min at −20°C (Nagaso et al. 2001). Permeabilized embryos were washed twice with PBT (phosphate-buffered saline+0.1% Tween) and postfixed for 25 min in 5% formaldehyde in PBT.

Embryos were hybridized with digoxigenin (DIG)-labeled *Dll* probe and a biotin-labeled *wingless* (*wg*) probe (used for staging and orientation). The *Dll* probe was transcribed from an approximately 2 kb fragment from the third *Dll* intron amplified from genomic DNA (using the primers 5'-GAATCTGGCGGTCTAGAGAAC and 5'-ACCGAGAACATTTGGCAGTC) and cloned into the pCR II vector (Invitrogen, Carlsbad, CA, USA). The resulting plasmid was cut with *HindIII* (NEB, catalog # R0104S) and anti-sense RNA was transcribed using the T7 promoter. DIG-labeled UTP nucleotides (Roche, Indianapolis, IN, USA, Cat #11277073910) were incorporated during transcription about once every 20 nucleotides. DIG haptens were detected with a sheep α-DIG antibody (Roche, catalog # 11333089001, 1:800 dilution) and a donkey α-sheep antibody (1:400 dilution) conjugated to Alexa647 fluorophores (Invitrogen, catalog #A-21448). Biotin haptens were detected with a mouse anti-biotin antibody (Roche, catalog #1297597, 1:800) and a donkey anti-mouse antibody (1:400) conjugated to Alexa488 fluorophores (Invitrogen, catalog #A-21202). UBX-HA proteins were detected with a rabbit α-hemagglutinin (HA) primary antibody (Invitrogen, catalog #71-5500, 1:100) and a donkey α-rabbit antibody (1:400) conjugated to Alex-

a555 fluorophores (Invitrogen, catalog #A-31572). Embryos were mounted in Prolong Antifade (Invitrogen, catalog # P-7481).

### Quantification of *Dll* transcripts and UBX protein concentration

Mid stage-11 embryos were selected and scanned using a Leica TCS SP2 AOBS confocal microscope (Wetzlar, Germany) at non-saturating intensity levels, using identical instrument settings for all embryos. Limb fields were scanned deep enough to capture all *Dll* mRNA signal (approximately 15  $\mu$ m). Where applicable, UBX-HA antibody signal was also detected. *Dll* transcription levels were determined as follows: *Dll* image stacks were collapsed to create single images. The anterior portions of each limb field were cropped and quantified by determining total fluorescent intensity. Eight limb fields were scored to determine the final average value. UBX protein levels were determined as follows: HA stacks were collapsed to obtain merged images and were cropped to the same dimensions as the *Dll* images. Average fluorescent intensity was determined using Velocity 4.0 (Improvision) by normalizing total fluorescence to stack thickness.

### Assaying Keilin's organ repression

Cloning of *abd-A* cDNA from *Artemia* was carried out as described in Ronshaugen et al. 2002. The coding sequence (GenBank accession #GQ141056) was cloned into the pUAST vector with the addition of a Kozak sequence at the 5' end, encompassing the start codon to ensure translation initiation and sequences encoding a HA tag at the 3' end to allow for detection of the transgene product. *Art* UBX(1-7) and *Art* UBX(7) mutations were introduced using PCR. These constructs were injected into *w*<sup>1118</sup> embryos and stably integrated via P-element transformation. Multiple transgenic lines of each construct were obtained and homozygous lines established. Ectopic expression was induced using the GAL4/UAS system (Brand and Perrimon 1993) by crossing the UAS-lines to flies carrying *arm-GAL4*. Use of this driver allowed for proper timing of expression starting at stage 9, before expression of endogenous *Dll*, to assay effects on limb formation (Sánchez-Herrero et al. 1994). To determine the levels of protein expression, 4-8 h embryos collected at 25°C were fixed for 20 min at RT. For *Ubx* constructs, a standard line ectopically expressing *Drosophila* UBX at 83  $\pm$  6% of endogenous levels (Tour et al. 2005) as used.

To establish a standard line for the *abd-A* transgenes, embryos from lines ectopically expressing *Drosophila* ABD-A and wild-type embryos (progeny of *w*<sup>1118</sup> crossed to *arm-GAL4*) were stained with an  $\alpha$ -*Drosophila* ABD-A mouse monoclonal antibody, mAbDMabd-A subclone 6A8.12 (Kellerman et al. 1990) at 1:500. Regions corresponding to the ventrolateral position of *Dll* expression of stage 11 embryos (determined morphologically) were measured for average luminosity in abdominal segments 2 and 3 for wild type and in all three thoracic segments for ectopic *abd-A* lines. Three *Drosophila abd-A* lines with an average luminosity near endogenous levels were identified.

For quantification of all other ectopically expressed *abd-A* lines, a *Drosophila abd-A* line was used as the standard (98  $\pm$  11%). Ectopic proteins were subsequently detected using a rat  $\alpha$ -HA antibody (clone 3F10, Roche, catalog # 11867423001). The average luminosity in regions of the thoracic segments corresponding to the

ventrolateral position of *Dll* expression in stage 11 embryos was calculated and reported as percentage endogenous protein expression.

To determine Keilin's organ repressive ability, cuticles were collected and cleared for phenotypic analysis (Wieschaus and Nüsslein-Volhard 1986). For each line, all three thoracic segments of 35 cuticles (210 possible Keilin's organs) were scored for presence or absence of Keilin's organs.

### Hatch assays

Ten to 20 males of the UAS-transgenic line were crossed to 20-25 virgin females carrying the *arm-GAL4* driver. Progeny from the crosses were collected for 24 h and counted. After aging for 24 h, unhatched embryos were counted to determine the number of embryos hatched. This number was normalized relative to a cross of *w*<sup>1118</sup> to *arm-GAL4* carried out in parallel. All available lines of each truncation were tested for viability, along with all available lines carrying the full-length *Artemia abd-A* and *w*<sup>1118</sup> as a control.

### Artemia husbandry

*A. franciscana* cysts (San Francisco Bay Brand, Newark, CA, USA) were hatched (day 1) in 11 15% Instant Ocean (Aquarium Systems, Mentor, OH, USA, catalog # SS3-50) with continuous light and aeration overnight. Temperature of the culture was maintained between 28°C and 32°C. Early development progressed at about one molt per day under these conditions until stage L4. For L1-L4 mixed stage collections, 0.3 g dehydrated cysts were hatched on day 1, an additional 0.2 g cysts added on days 2 and 3, 0.1 g cysts added on day 4. Live animals were collected on day 5. To grow *Artemia* to late larval stages, 250  $\mu$ l of diluted Tahitian Blend algal paste (Brine Shrimp Direct, Ogden, UT, USA) were added every other day starting on day 3. Animals were collected and fixed once they developed to the desired stage.

### Antibodies

An  $\alpha$ -*Artemia* ABD-A antibody was made against a GST-fusion of the N-terminal domain, up to and including the YPWM motif of *Artemia* ABD-A (Pocono Rabbit Farm and Laboratory Inc., Canadensis, PA, USA). The rabbit polyclonal sera were affinity purified through a Quickpure column (Sterogene, Carlsbad, CA, USA, catalog # QP01-01) and tested for specificity on *Drosophila* embryos at 1:200 dilution. The 4F11  $\alpha$ -EN antibody (Patel et al. 1989) was used at 1:30 dilution. The FP6.87 antibody (Kelsh et al. 1994) was concentrated four- to fivefold using Centricon filter units (Millipore, Billerica, MA, USA), then used at 1:2 dilution.  $\alpha$ -*Drosophila* ABD-A (Kellerman et al. 1990) was used at 1:400. Rat  $\alpha$ -HA was used at 1:500 dilution (Roche, catalog # 11867423001).

### Artemia immunohistochemistry

*Artemia* were fixed based on protocols supplied by N. Patel (personal communications) with modifications. 0.2 g of live L1-L4 *Artemia* or 400-500  $\mu$ l late-stage animals were fixed in 33 ml 0.1 M PIPES, 2 mM EGTA, 1 mM MgSO<sub>4</sub>, 3.4% formaldehyde (ULTRAPURE ampules, Polysciences, Warrington, PA, USA, catalog #18814) for 5 min. Tween-20 was added to 0.02% and fixation continued for another 3 min. The *Artemia* were subsequently dehydrated using a stepwise transfer into methanol and stored at

–20°C until ready to proceed with the immunodetection. Antibody staining was carried out as described for *Drosophila* embryos in Kosman et al. (2004) with the addition of a sonication step before blocking. Sonications were carried out using a Branson Sonifier 150 (BLD Inc., Garner, NC, USA) at a maximum output of 5 W in 40 ml PBT. Total sonication time was stage dependent. One hundred microliters of L1–L4 stage *Artemia* were sonicated for a total of 16 sec, seven to eight L9–L10 stage animals for 50 sec and seven to eight L11–L12 stage animals for 62 sec. Two second bursts of sonication were followed by inversion of the sample several times to mix the animals thoroughly between bursts until the total sonication time was achieved. For detection of nuclei to aid in staging, DAPI was added to the mounting media. Animals were mounted ventral side up in 2.5% w/v DABCO (Sigma #D-2522, St. Louis, MO, USA), 50 mM Tris pH 8.0, 90% glycerol. Images were obtained using a Leica TCS SP2 AOBS confocal microscope. Though dilutions worked out for staining *Drosophila* embryos using the 4F11 antibodies and FP6.87 antibodies also worked well in *Artemia*, for *Artemia* stains, the rabbit  $\alpha$ -*Artemia* ABD-A was used at the higher concentration of 1:100 to ensure that the inability to detect protein during early stages was not due to insufficient levels of antibodies.

### In situ hybridization

For *Artemia* in situ hybridizations, probes to the first 731 bp of coding sequence for *Artemia* EN and to the complete coding sequence of *Artemia* *Ubx* and *abd-A* were prepared as described in Kosman et al. (2004). *Artemia* were fixed in the same manner as for immunohistochemistry, then hydrated using a MeOH:PBT series and sonicated as described previously. In situ hybridizations were carried out based on *Drosophila* protocols above with the following modifications: *Artemia* were not treated with xylenes, Protease K treatment was carried out for 2 min at a final concentration of 5  $\mu$ g/ml (G. Boekhoff-Falk, personal communications), the transfer to hybridization solution before the prehybridization step was carried out with reagents preheated to 55°C and included an extra 5 min wash with hybridization solution at 55°C. The hybridization solution was modified with an addition of SDS to a final concentration of 1% as suggested by N. Patel (personal communications). Probes were hybridized for 19–19.5 h at 55°C. After hybridization, animals were transferred to PBT using a graded hybridization solution/PBT series preheated to 55°C. Subsequent washes and antibody incubations were carried out at room temperature or 4°C if carried out overnight. Detection of probes required tyramide amplification (TSA Plus, Perkin Elmer, Waltham, MA, USA, catalog # NEL744001KT, NEL741001KT) at 1:75 for 15 min at RT with occasional mixing. Tyramides were resuspended in water instead of DMSO. DIG probes were detected with Cy3 tyramide and FITC probes with FITC tyramide using sequential tyramide reactions (Kosman et al. 2004) and a posthybridization fixation in 1% formaldehyde for 5 min followed by two rinses and three 5-min washes with PBT was added before antibody detection. Mounting was carried out as described above for immunohistochemistry. Images were obtained using a Leica TCS SP2 AOBS confocal microscope and deconvolved using the AutoDeblur software (MediaCybernetics).

*Drosophila* embryo in situ hybridizations and immunohistochemistry were carried out as described in Kosman et al. (2004).

### RT-PCR and quantitative PCR

L4 stage *Artemia* were collected by hatching 0.5 g cysts and on day 2 collecting L1 nauplii as described previously. The L1 nauplii were transferred to fresh 15% Instant Ocean then light and aeration were continued until day 5. On day 5, animals were collected by taking advantage of their positive photo-tactic behavior. In this manner, approximately 98% of the animals collected were stage L4 larvae.

A sample of each set of collected animals was taken and the developmental stage confirmed under a dissecting scope. The collected animals were flash frozen in liquid nitrogen and stored in 50-mg aliquots at –80°C. The following were all carried out per the manufacturer's instructions: (1) RNA was extracted using 1 ml Trizol reagent (Invitrogen, catalog #15596-018) per 50 mg L4 *Artemia*. (2) PolyA RNA was purified using the Oligotex mRNA kit (Qiagen, Valencia, CA, USA catalog #70042) then treated with DNase (Ambion, Austin, TX, USA, catalog # AM2222). (3) cDNA were generated using the Ambion RETROscript kit (catalog #, AM1710) and (4) treated with RNase A (Ambion, catalog # AM2270).

RT-PCR amplification of the resulting cDNA was carried out using the following primers for amplification of *Artemia* *abd-A*: 5'-CCCAAATGGTTGTCCTCG-3' and 5'-GTCCATCATTCCTCAGGTG-3' and for amplification of *Artemia* *Ubx*: 5'-ATG AATTCGTATTTTGAACAGAATGG-3' and 5'-AAGCTTTT CATCTTTTTCATCGTCACT-3'. Cycling conditions used were 95°C for 5 min followed by 35 cycles at 94°C for 30 sec, 50°C (*abd-A*) or 55°C (*Ubx*) for 30 sec, 72°C for 1 min then 72°C for 5 min.

Quantitative PCR was carried out using the ABI Prism 7000 System using 5  $\mu$ l cDNA, 2.5  $\mu$ l each of 600 nM primers and 10  $\mu$ l Power Sybr Green PCR Master Mix (ABI, Foster City, CA, USA, catalog #4367659) on six 10<sup>–1</sup> serial dilutions of cDNA template using the default cycling conditions to generate a standard curve. The dissociation protocol was run to determine whether more than one product was generated. Primers used for amplification of *Artemia* *abd-A* were 5'-CGTCTATGGCTACAGCAGCA-3' and 5'-TTCGAAGGGTCATTGAAGC-3' and for *Artemia*  $\alpha$ -tubulin, the primers used were 5'-GAAAGTACGTGGCCTGCTG-3' and 5'-GCATTGACGTCTTTTGGTACG-3'-3'. Reactions were carried out in duplicate.

## RESULTS

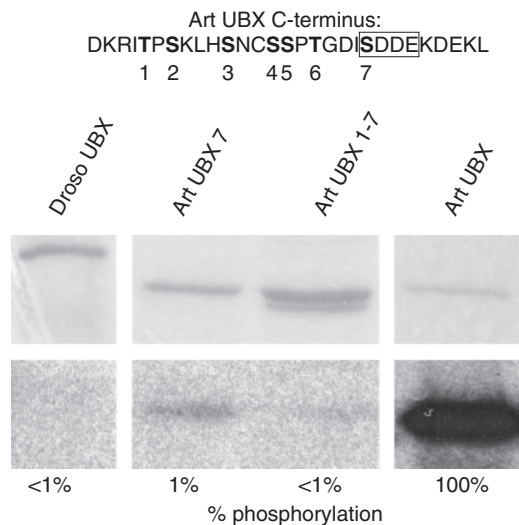
### The Serine within the *Artemia* UBX C-terminal CK2 consensus sequence is a major in vitro CK2 phosphorylation site

In insects, the *Hox* gene *Ubx* is expressed in the anterior abdominal primordia of embryos, where it partially or completely represses limb development. In developing *Artemia*, *Ubx* transcripts and protein are expressed throughout the trunk, but *Dll* is still expressed in limb buds and large limbs develop. Ronshaugen et al. (2002) found that Serine (Ser) and Threonine (Thr) residues in the C-terminus of *Artemia* *Ubx* partially inhibited its limb repressive function. One of these Ser residues is in a consensus phosphorylation site for CK2. It seemed plausible that the consensus CK2 site in *Artemia* UBX

might inhibit its limb repressive function, as mutation of four CK2 sites in the *Drosophila Hox* protein ANTP changed it into a limb repressor in embryos (Jaffe et al. 1997). The *Artemia* UBX protein has a single consensus CK2 site in C-terminal sequences, but whether this site is an actual substrate for CK2, and whether it is necessary or sufficient to inhibit *Artemia* UBX limb repressive function, has not been tested.

CK2 kinase assays were performed to determine whether *Artemia* UBX is actually phosphorylated within the C-terminus consensus CK2 site. The substrates and controls in the kinase assays were GST-UBX fusion protein variants that were produced in *E. coli*. These included fusion proteins with wild-type *Drosophila* UBX-1a, wild-type *Artemia* UBX, and two mutant versions of *Artemia* UBX with Ala substitutions in C-terminal Ser/Thr residues (Fig. 1).

The levels of CK2 phosphorylation for the UBX proteins was determined by scanning autoradiographed gels that were loaded with kinase reactions ("Materials and Methods"), then compared with *Artemia* UBX (set at 100%), after normalizing by the amount of protein per reaction. The percentage of phosphorylation in Fig. 1 is the average from two separate



**Fig. 1.** In vitro CK2 phosphorylation occurs mainly at the consensus CK2 site within the C-terminus of *Artemia* UBX. Sequence of the C-terminus of *Artemia* UBX is shown at the top with the Serines/Threonines in bold type and numbered. The CK2 consensus site is boxed. GST-fusion protein constructs were purified and in vitro phosphorylation reactions carried out. GST-*Art* UBX was strongly phosphorylated by CK2 and the amount of phosphorylation set as the standard at 100%. CK2 phosphorylation for all other proteins was calculated relative to GST-*Art* UBX. There was no detectable phosphorylation of GST-*Drosophila* UBX by CK2. GST-*Art* UBX with all Serines and Threonines mutated to Alanine (*Art* UBX 1-7) showed almost no phosphorylation by CK2 in vitro and GST-*Art* UBX with mutation of the Serine within the CK2 consensus site to Alanine (*Art* UBX 7) showed a similar level of reduced phosphorylation.

experiments. GST alone was not phosphorylated by CK2 (data not shown). *Artemia* UBX but not *Drosophila* UBX was strongly phosphorylated by CK2 (Fig. 1). Mutation of only the Ser within the CK2 consensus site (*Art* UBX 7, Fig. 1) abolished almost all phosphorylation of *Artemia* UBX. Mutation of additional Ser/Thr residues in the C-terminus showed only an additional modest reduction of phosphorylation levels (*Art* UBX 1-7, Fig. 1).

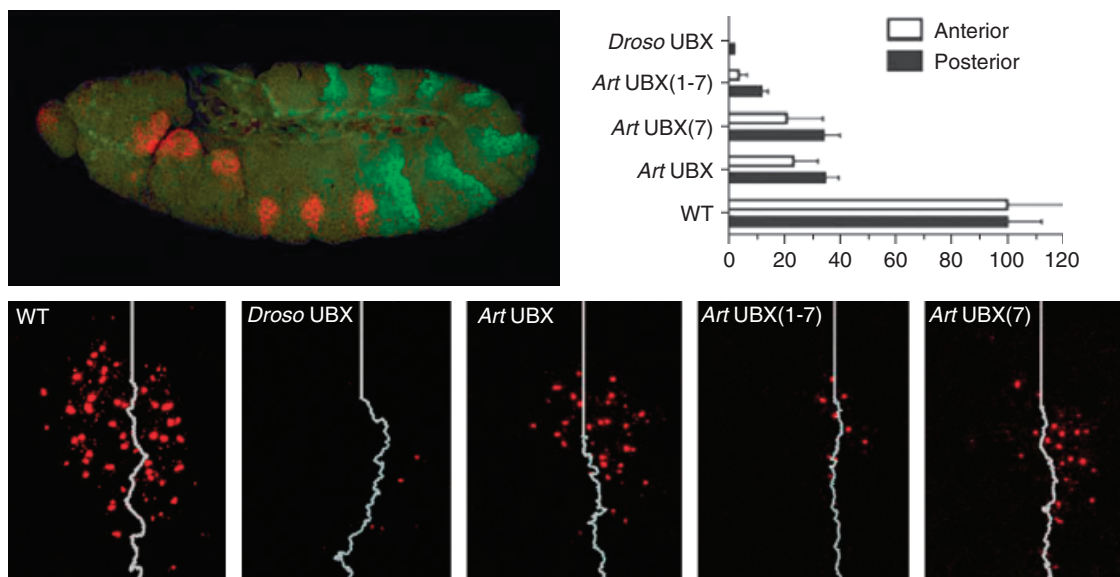
To test whether the Ser in the CK2 site of *Artemia* UBX inhibited limb repressive function, we compared the effects of ectopic expression in *Drosophila* embryos of the proteins we tested in the in vitro CK2 assays. The UAS-GAL4 system (Brand and Perrimon 1993) was used to express the UBX proteins at or near endogenous levels, and the effects of the proteins on limb development were assayed by quantifying the levels of *Dll* transcripts in the first thoracic segment (T1) of stage 11 *Drosophila* embryos (Fig. 2). The average level of *Dll* transcript signal in wild-type embryos was set to 100%.

*Drosophila* UBX completely repressed *Dll* in the anterior compartment of T1, with only occasional transcript signals detected in the posterior compartment. Wild-type *Artemia* UBX repressed *Dll* to 20–40% of wild-type levels, with a stronger repressive effect in the anterior compartment of T1 as compared with the posterior compartment (Fig. 2). When all C-terminal Ser/Thr residues were mutated to Ala, the repressive effect of *Artemia* UBX on *Dll* was increased significantly, close to the levels observed for *Drosophila* UBX. However, mutation of only the Ser within the CK2 consensus site (*Art* UBX 7) did not increase *Artemia* UBX repressive function. We conclude that although Ser and Thr residues in the C-terminus of *Artemia* UBX reduce its ability to repress *Dll* transcription, the Ser residue in the CK2 site is not crucial for this reduction of repressive ability in embryos.

### ***Artemia* ABD-A protein expression is not produced in *Drosophila* embryos unless the C-terminus is truncated**

While the reduced repressive function of *Artemia* UBX on *Dll* expression can help to explain how limbs develop in the *Artemia* trunk, whether *Artemia* ABD-A also has a similar reduced repressive function was unknown. Averof and Akam (1995) suggested that *Artemia* ABD-A protein was present in limb-bearing trunk segments, but this was largely based on the use of a monoclonal antibody, FP6.87, which detects a conserved epitope shared by both UBX and ABD-A (Kelsh et al. 1994; Averof and Akam 1995). The previous study left open many possibilities, including that *Artemia* ABD-A protein might be produced at levels so low that limb development was not repressed, or that *Artemia* ABD-A protein function had evolved to have reduced limb repressive ability.

To test the embryonic/larval limb repressive ability of *Artemia* ABD-A, we constructed transgenic *Drosophila* lines



**Fig. 2.** *Artemia* UBX with all Serine/Threonine sites within the C-terminus mutated to Alanine represses *Dll* expression in *Drosophila* embryos. Wild-type (WT) stage 11 embryos express *Dll* transcript (red) encompassing encompassing a stripe of *wg* expression that demarcates the anterior field from the posterior field. *Abd-A* protein staining is shown in green. The white line marks the separation of the anterior and posterior fields of *Dll* expression (as determined by *wg* expression, data not shown). Stage 11 embryos ubiquitously expressing *Drosophila* UBX, *Artemia* UBX or *Artemia* UBX with Serine/Threonine to Alanine mutations in the C-terminus show varied abilities to repress *Dll* expression. Mutation of all Serines/Threonines within the C-terminus of *Artemia* UBX, *Art* UBX (1–7), results in strong repression of *Dll*. While mutation of the Serine within the CK2 consensus site only, *Art* UBX (7), results in a level of *Dll* expression comparable to wild-type *Artemia* UBX.

carrying an *Artemia abd-A* full-length protein coding sequence fused to a HA tag (Fig. 3A), under the control of the GAL4-UAS system. To our surprise, after inducing expression of an *Artemia* ABD-A-HA cDNA in early embryos, the larvae develop into viable and fertile adults with normal limbs. To test whether the transgenic strains with the inducible *Artemia* cDNAs produced *abd-A* messenger RNA, we carried out in situ hybridizations using an anti-sense probe to the entire ABD-A coding sequence. Ubiquitous high levels of cytoplasmic transcripts were detected in lines containing the full-length *Artemia* ABD-A-HA fusion (Fig. 3B). We next tested whether *Artemia* ABD-A protein was being produced in transgenic embryos using an  $\alpha$ -HA antibody and immunofluorescence assays. No *Artemia* ABD-A-HA protein was detected during embryogenesis, in any tissues, including the central nervous system (Fig. 3B). We concluded that either the ABD-A mRNAs were not being translated into protein, or that the resulting proteins were highly unstable.

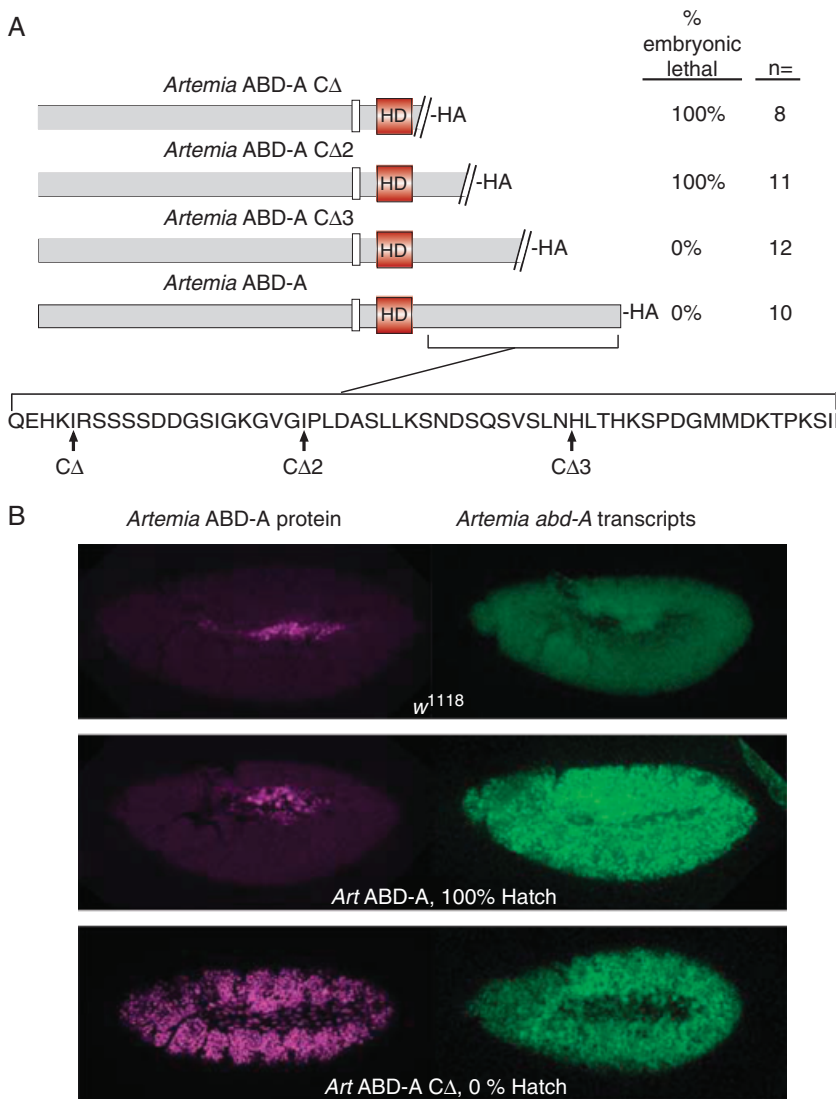
The *Artemia* ABD-A-HA coding region was cloned into an expression vector with the open reading frame flanked with a 5' UTR from *Drosophila hsp-70* and a 3' UTR from SV40. Protein expression from similar expression constructs containing numerous coding regions have been induced in *Drosophila* embryos, both in our lab and others. We decided to test whether the *Artemia* ABD-A coding sequences contained regions that prevented protein accumulation in *Drosophila* embryos.

A deletion of *Artemia* ABD-A C-terminal protein coding sequences identified a region that prevents translation of the *abd-A* transcripts or accumulation of ABD-A proteins. This deletion mutant, ABD-AC $\Delta$ , produced abundant amounts of both *Artemia* ABD-A transcripts and proteins in *Drosophila* embryos (Fig. 3B), and resulted in embryonic lethality (Fig. 3A). A test of smaller deletion mutants suggested that the proposed translational inhibition or protein instability signal resides in the 18 amino acid coding sequence between the CA2 and CA3 endpoints (Fig. 3A).

### Truncated *Artemia* ABD-A represses Keilin's organs in *Drosophila* embryos and is capable of homeotic function

The ability of ABD-AC $\Delta$ , the stable version of *Artemia* ABD-A, to specify abdominal segment morphology and repress larval limb development was assayed in *Drosophila* embryos. The vestigial *Drosophila* larval limbs are called Keilin's organs, and they develop from clusters of embryonic neuroectodermal cells that express *Dll* (Cohen and Jürgens 1989). *Artemia* ABD-AC $\Delta$  was capable of completely repressing Keilin's organs when expressed at levels near endogenous *Drosophila* ABD-A levels (Fig. 4A). However, when *Artemia* ABD-AC $\Delta$  protein expression levels fell even 20% below endogenous *Drosophila* ABD-A levels, its limb





**Fig. 3.** Protein expression of *Artemia* ABD-A cannot be induced in *Drosophila* embryos unless C-terminal sequences are deleted. (A) Hatch assays of various *Artemia* ABD-A truncations. The truncation point for each transgene is shown at the bottom of the panel. Hatch percentages were calculated relative to *w<sup>1118</sup>*. Ubiquitous expression of *Artemia* ABD-A protein showed the expected embryonic lethal phenotype only for truncated proteins *Artemia* ABD-A CΔ and *Artemia* ABD-A CΔ2. (B) *Drosophila* embryos induced to ubiquitously express *Artemia abd-A* transgenes were tested for the presence of transcript and protein. The top panel shows the negative control of embryos from arm-GAL4 crossed to *w<sup>1118</sup>*. Transcripts were detected in embryos expressing both full-length and truncated ABD-A, but protein was only detected for embryos expressing the truncated *abd-A* transgene.

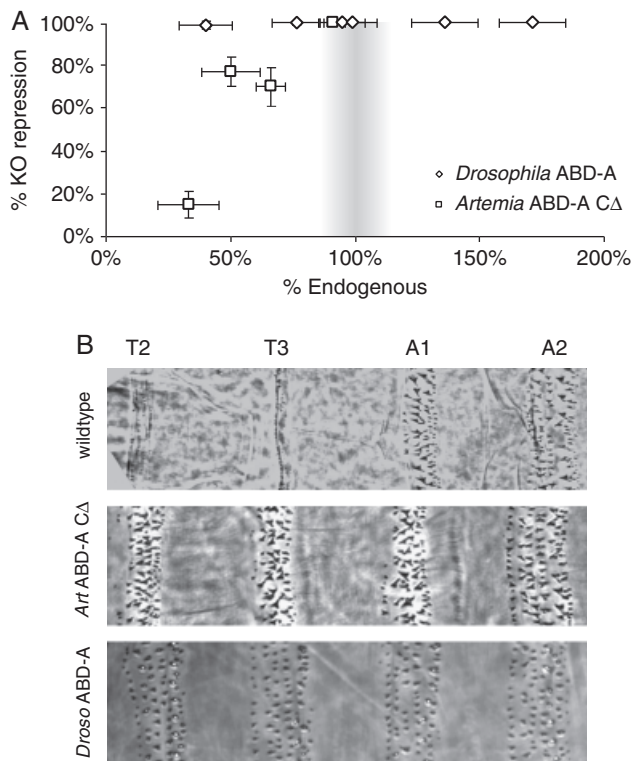
repressive ability was reduced. When *Artemia* ABD-ACA protein was expressed at approximately 35% of endogenous *Drosophila* ABD-A levels, limb repressive function was nearly absent. However, *Drosophila* ABD-A did not display a concentration sensitivity with regard to limb repression. In ectopic expression assays, Keilin's organs are fully repressed even when *Drosophila* ABD-A protein is expressed at approximately 40% of endogenous levels (Fig. 4A).

Cuticles of *Drosophila* embryos ectopically expressing *Artemia* ABD-ACA and ranging in protein expression from 30% to 90% of endogenous levels were analyzed in parallel with the standard line ectopically expressing *Drosophila* ABD-A at 100% of endogenous levels. In cuticles from all *Artemia* ABD-ACA lines analyzed, we observed the same phenotypes described for ectopic expression of *Drosophila* ABD-A, such as head involution defects, formation of denticle belts in the

head and repression of the denticle "beard" characteristic of segment T1 (data not shown). However, in contrast to ectopic *Drosophila* ABD-A, which transforms thoracic and abdominal segments toward A2–4 segment identities (Sánchez-Herrero et al. 1994), *Artemia* ABD-ACA transformed thoracic denticle belts toward abdominal segment 1 (A1-like) identities (Fig. 4B).

### Trunk development and gene expression in *A. franciscana*

Finding no accumulation of full length *Artemia* ABD-A protein in *Drosophila* embryos led us to wonder if a similar phenomenon occurred in developing *Artemia*. *Artemia* embryogenesis begins within the reproductive tract of fertilized females. Under permissive conditions, embryogen-



**Fig. 4.** Truncated *Artemia* ABD-A represses Keilin's organs and transforms segments toward an abdominal identity in *Drosophila* embryos. (A) *Drosophila* ABD-A can completely repress Keilin's organs even when ectopically expressed at approximately 40% of endogenous levels. *Artemia* ABD-ACΔ can repress 100% of Keilin's organs when ectopic expression is near endogenous levels, but shows reduced ability to repress Keilin's organs at lower protein concentrations. (B) When ubiquitously expressed in *Drosophila* embryos, *Artemia* ABD-ACΔ transforms thoracic denticle belts toward an abdominal A1-like identity while *Drosophila* ABD-A transforms denticle belts toward abdominal A2–4 identities.

esis progresses and results in the live birth of first instar larvae, also known as L1 nauplii, into the surrounding sea water. In the L1 stage, the anterior head structures have already begun differentiation, but the gnathal and trunk region grow and differentiate in an anterior–posterior gradient of development during the 14 larval stages (Schredhardt 1987). New segments develop from a growth zone that resides at the posterior end of the trunk. *Artemia* develops 11 trunk segments each bearing a pair of limbs. Trunk segmentation begins with the formation of segmentation furrows followed by limb bud formation (Fig. 5A). The developing limbs undergo articulation, at which point the limbs are known as thoracopods. After articulation, the thoracopods differentiate so that within each limb several podites develop and setae form and elongate. *Artemia* are staged by the number of developing thoracopods and by the degree of limb differentiation (Schredhardt 1987).

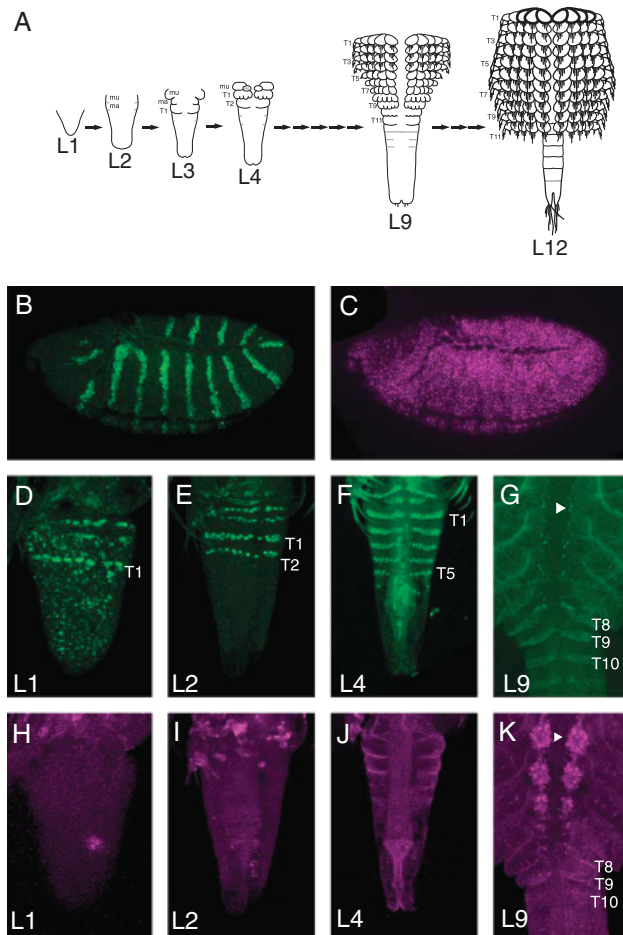
### ABD-A protein is detected in the trunk CNS but not limb primordia of developing *Artemia*

To test if ABD-A protein accumulates in the trunk epidermis of early-stage *Artemia* before and during limb differentiation, we developed and affinity purified polyclonal antibodies to the N-terminal region of *Artemia* ABD-A. The specificity of these antibodies was tested on *Drosophila* embryos ectopically expressing various HOX proteins. The antibodies detected *Artemia* ABD-A specifically and did not cross-react with *Artemia* UBX, *Drosophila* ABD-A or *Drosophila* UBX proteins (data not shown).

Double stains using  $\alpha$ -engrailed antibody ( $\alpha$ -EN; Patel et al. 1989) plus  $\alpha$ -*Artemia* ABD-A (Fig. 5) and double stains with  $\alpha$ -UBX/ABD-A (FP6.87) plus  $\alpha$ -*Artemia* ABD-A were carried out on various stages of *Artemia* larvae (see supporting information Fig. S1). Because of the difficulty in permeabilizing *Artemia* larvae, which have a cuticular exoskeleton, a variable fraction in each experiment exhibited antibody staining. The segmental staining pattern of EN was used as a larval stage marker and a positive control for antibody permeability of the *Artemia* larvae. Early-stage *Artemia* nauplii (L1–L4), and stage 11 *Drosophila* positive control embryos ubiquitously expressing truncated *Artemia* ABD-A (*Artemia* ABD-ACΔ, were processed together after separate fixation methods (“Materials and Methods”). Because ABD-A protein expression accumulates in the neuromeres of late-stage *Artemia* (Averof and Akam 1995), L9–L12 stage *Artemia* were also stained as a positive control with the  $\alpha$ -*Artemia* ABD-A antibodies (Fig. 5K). As a negative control, *Artemia* larvae were stained with an  $\alpha$ -*Drosophila* ABD-A antibodies (data not shown).

EN and *Artemia* ABD-ACΔ proteins were both detected in *Drosophila* control embryos induced to ubiquitously express *Artemia* ABD-ACΔ (Fig. 5, B and C). In *Artemia* larvae, the EN protein expression pattern could be detected in approximately 70% of the L1–L4 stage animals (Fig. 5D–F). But, ABD-A protein could not be detected in any L1–L4 stage larvae in which the EN control staining was detected (Fig. 5H–J). In late-stage *Artemia* larvae, ABD-A expression could be detected within midline neuromeres (Averof and Akam 1995; Fig. 5K). In L9 stage animals, ABD-A neuromere expression is seen in trunk segments that have already developed thoracopods (e.g., T8 and more anterior trunk segments), but not in segments that are less differentiated (e.g., T9 and T10, Fig. 5K). At this stage, EN protein expression in midline neuromeres can also be detected at roughly the same anterior–posterior position (T9, Fig. 5G). Similar double stain experiments were also carried out with  $\alpha$ -*Artemia* ABD-A antibodies plus FP6.87 control antibody controls. In L1–L4 stage larvae where FP6.87 staining was detected throughout the trunk, a specific ABD-A signal was never detected (supporting information Fig. S1).





**Fig. 5.** EN and ABD-A protein expression in *Artemia*. EN is in green and ABD-A is in magenta. Anterior is left for *Drosophila*, up for *Artemia* this and subsequent figures. (A) Trunk development in selected *Artemia* larval stages. The gnathal segments are shown in the L2 and L3 stages, otherwise head structures are not represented in these schematics. After the first molt, segmentation begins in the two gnathal segments (mu and ma). Additional thoracic segments emanate from the posterior growth zone, producing an anterior–posterior gradient of development with anterior segments at a more advanced stage of differentiation than the posterior segments. Thoracomers, trunk segments with limb buds, mark the start of limb formation (e.g., T1 of L3 stage *Artemia* or T11 of L9 stage *Artemia*). As limb development proceeds, articulation begins and the trunk segments are now known as thoracopods (e.g., T1 of L4 stage or T10 of L9 stage). The thoracopods undergo elongation and further differentiation so that several podites form for each limb and setae develop and elongate. (B, C) L1–L9 mixed stages of *Artemia* were stained with *Drosophila* embryos ectopically expressing *Artemia* ABD-ACΔ. Detection of EN protein (B) and *Artemia* ABD-ACΔ protein (C) in *Drosophila* embryos at stage 11 are shown. In *Artemia*, EN stripes (which mark the posterior of each segment) are detected in early stages (D, E, F). G and K shows the midline of a stage L9 *Artemia*. By the L9 stage (G), EN protein is also detected in a few cells in the neuromeres of each segment (arrowhead). ABD-A protein is not expressed to detectable levels in early stages (H, I, J) but is clearly expressed in neuromeres at stage L9 (K, arrowhead). In the L9 stage (G, K) limbs of trunk segments anterior to T6 are fully differentiated. T6 thoracopods have almost finished differentiation. T8 thoracopods have started articulation (vertical furrows visible in K) and T9 thoracomers are just about to initiate articulation. ABD-A protein expression in neuromeres have initiated in a few cells in the T8 trunk segment and within all the neuromere cells in the fully differentiated trunk segments from T6 to T1. mu: maxillary segment, ma: maxillary segment.

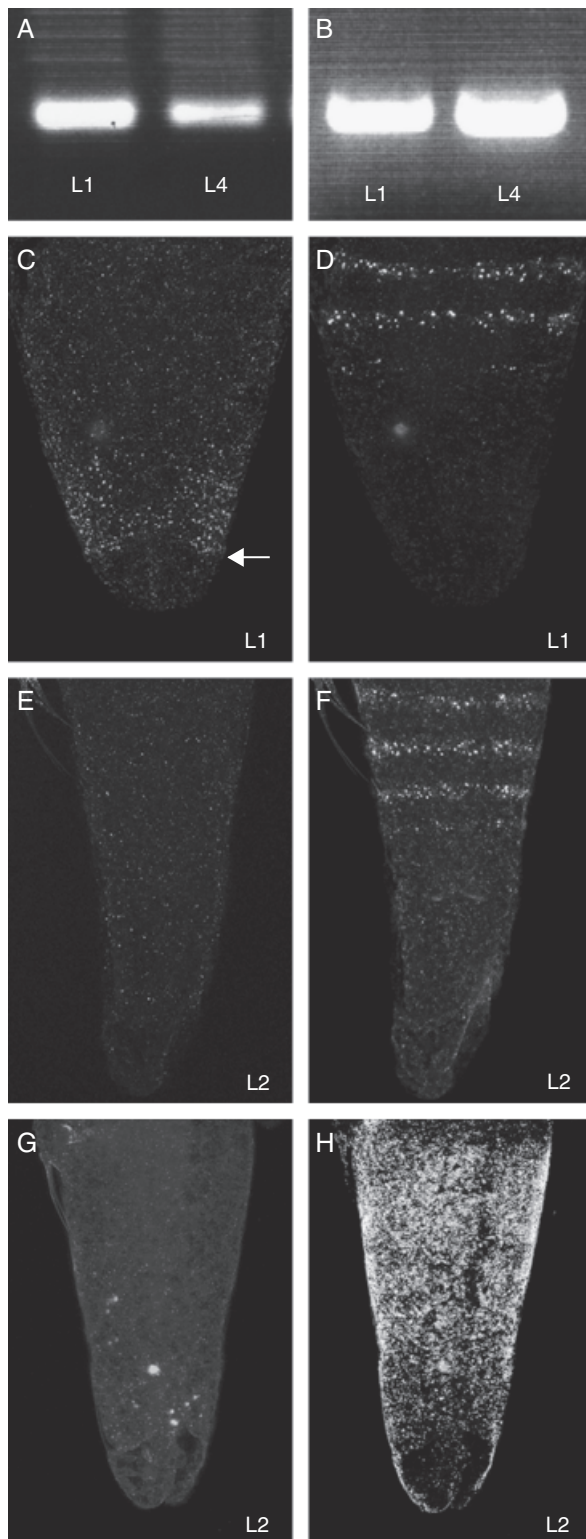
### ***abd-A* transcripts are expressed at low levels in early *Artemia* epidermis**

As a first test of whether *abd-A* transcripts were expressed in early larval stages, we carried out RT-PCR. Poly-A RNA from L1 and L4 stage *Artemia* larvae was purified and reverse transcribed, and PCR was carried out using primers specific to *abd-A* and *Ubx*. Single primer controls did not amplify any bands (not shown). PCR products of the expected sizes for both *Ubx* and *abd-A* were detected at both L1 and L4 stages, and DNA sequencing confirmed that the RT-PCR fragments corresponded to *Artemia abd-A* transcript sequence (Fig. 6, A and B).

To determine the expression pattern of *abd-A* transcripts in early-stage *Artemia*, we carried out pairwise in situ hybridizations with *en*, *Ubx*, and *abd-A* probes on L1–L4 stage *Artemia*. Sense probe controls were also performed in parallel. As with protein detection, the efficiency of RNA detection is influenced by variable permeabilization of *Artemia* larval cuticle. Control RNA signals were detected in approximately 60% of fixed and hybridized *Artemia* larvae. At the L1 stage,

an *abd-A* transcript signal could be detected within the growth zone in approximately 25% of larvae staining for *en* (Fig. 6, C and D). In stage L2–L4 stage animals, a localized *abd-A* signal was not consistently detected by in situ hybridization in larvae that stained with the *en* probe, suggesting that *abd-A* transcripts were at very low levels (Fig. 6, E and F). Similar results were obtained with later stage larvae doubly stained for *Ubx* and *abd-A* transcripts. *Ubx* signal was detected in a region matching the staining pattern obtained with FP6.87 antibodies and antiserum specific for *Artemia* UBX (Averof and Akam 1995; Averof and Patel 1997; Shiga et al. 2006), but after the L1 stage a localized *abd-A* transcript signal was too weak to be consistently detected (Fig. 6, G and H).

As a further test of the presence of *abd-A* transcripts at L4 state, because in situ signals were not consistently detected, we carried out quantitative PCR (qPCR). Primers were made to both *Artemia abd-A* and *α-tubulin*. To ensure that the products generated were due only to amplification of transcripts, samples were treated with DNase after isolation of polyA-RNA and treated with RNase after reverse transcription.



**Fig. 6.** *abd-A* transcript is transcribed at low levels in the *Artemia* trunk of early-stage larvae. RT-PCR amplified both *Artemia Ubx* (A) and *Artemia abd-A* (B) in stage L1 and L4 larvae. Double in situ hybridizations with *abd-A*-DIG (C, E) plus *en*-FITC (D, F) probes or *abd-A*-FITC (G) probes plus *Ubx*-DIG (H). The posterior boundary of the surmised growth zone is indicated by an arrow in C. *abd-A* transcript was detected in L1 larvae within the growth zone.

Control reactions with water only and without reverse transcription were negative. Dissociation curves generated after the reactions were complete showed only one product for each of the experimental samples. qPCR results indicate that, considered over the entire animal, *abd-A* transcript is between 1000-fold and 2400-fold less abundant than *α-tubulin* transcript. Because the *abd-A* transcript is likely to be produced in only small fraction of cells that produce *α-tubulin* transcripts (Fig. 6C), this indicates that *abd-A* transcripts are present at significant levels at both L1 and L4 states of development.

## DISCUSSION

All segments of the *Artemia* trunk develop limbs despite *Artemia* having the trunk *Hox* genes *Ubx* and *abd-A*. Our results suggest two reasons why these *Artemia* genes fail to repress limbs during development. First, although *Ubx* transcripts and proteins are expressed in limb buds of the *Artemia* trunk (Averof and Akam 1995, Shiga et al. 2006), Ser and Thr residues in the C-terminal region of *Artemia* *UBX* partially inhibit its repressive function on *Dll* (Fig. 1, and Ronshaugen et al. 2002). Second, *Artemia* *ABD-A* protein is not produced at detectable levels in developing early larval limb buds or other epidermal cells, even though *abd-A* transcripts accumulate at low levels. We conclude that *ABD-A* protein does not play a role in conferring epidermal morphological identity in developing *Artemia*.

This study reveals a novel variation in *HOX* function that is associated with evolution of body patterning. In *Artemia*, the loss of *ABD-A* *HOX* protein function during early-epidermal development is correlated with the development of a homonomous trunk, each of the segments bearing limbs. *ABD-A* protein does accumulate at later stages in cells that appear to be part of the central nervous system (Fig. 5K, and Averof and Akam 1995). Although there are examples of *Hox* genes whose expression patterns have been dramatically altered during evolution (Lohr and Pick 2005; Hughes and Kaufman 2002a,b; Hsia and McGinnis 2003), the known examples have involved genes such as *Drosophila bicoid* or *fushi tarazu*, which have also undergone dramatic changes in protein sequence and developmental function.

The finding that *Artemia* *ABD-A* protein is not expressed in the trunk epidermis is unexpected. In many arthropods, the

expression pattern of ABD-A protein during development has been inferred by staining with an antibody that recognizes a conserved epitope in both UBX and ABD-A, and correlating this pattern with the specific expression patterns of *Ubx* and *abd-A* transcripts (Averof and Patel 1997; Zheng et al. 1999; Abzhanov and Kaufman 2000a, b; Blin et al. 2003). Such studies have indicated that *abd-A* transcripts and protein are produced in a HOX-like pattern in the posterior trunk epidermis of many insects and two crustaceans, and the *abd-A* expression domain is correlated with partial or complete repression of limbs (Tear et al. 1990; Nagy et al. 1991; Shippy et al. 1998; Peterson et al. 1999; Abzhanov and Kaufman 2000a, b; Hughes and Kaufman 2002a, b; Zhang et al. 2005). Thus it seems likely that the absence of ABD-A protein expression in the epidermis of the developing trunk of *Artemia* is a derived condition in crustaceans. Whether other branchiopod crustaceans also lack ABD-A protein during epidermal development is as yet unknown.

Low levels of *abd-A* transcripts are detected in the epidermis in early *Artemia* larval stages of developing *Artemia* larvae. Thus it appears that the failure to accumulate abundant ABD-A protein in the developing *Artemia* trunk epidermis is partly mediated at the level of transcript production and/or stability. However, some regulation of *abd-A* at the level of translation of protein stability is apparently also occurring in early *Artemia* larvae as no protein is detected at any early-larval stage. This is consistent with the behavior of the *Artemia abd-a* cDNA after its activation in *Drosophila* embryos. *Artemia abd-A* transcripts can be induced to accumulate to high levels in *Drosophila* embryos, but full-length protein is not produced, even in the central nervous system. Our evidence indicates this is due to C-terminal sequences in the *Artemia* ABD-A protein coding region that either inhibit translation or result in extreme protein instability. A search for common protein motifs that promote protein instability (e.g., PEST sequences) in *Artemia* ABD-A C-terminal sequences did not result in any obvious matches. The lack of ABD-A protein expression in *Artemia*, then, appears to be the result of mechanisms occurring at both the transcriptional and translational levels.

### Evolution of *abd-A Hox* gene expression and effects on arthropod morphology

Two previous studies have suggested that molecular variation of *abd-A* expression has played a role in arthropod morphological evolution. One interesting example is in cirripedes (barnacles), where at least three species appear to have lost the *abd-A* gene despite retaining *Ubx* and *Abd-B* (Mouchel-Vielh et al. 1998; Blin et al. 2003; Deutsch and Mouchel-Vielh 2003). This missing abdominal *Hox* gene is correlated with the severe reduction or absence of abdominal segments in cirripedes. Another study involved the analysis of *abd-A* transcript expression patterns from two ant species with different

abdominal morphologies (Niculita 2006), finding a correlation between variations in *abd-A* transcript expression pattern during early development between two different ant species and variations in their abdominal segment morphologies.

### *Artemia* UBX limb repressive function

When expressed in *Drosophila* embryos at levels equivalent to endogenous UBX, Ser, and Thr residues in the C-terminus of *Artemia* UBX partially inhibit its ability to repress *Dll*. However, the single Ser in the *Artemia* UBX C-terminus that is phosphorylated by CK2 has no apparent inhibitory role. Although previous studies have shown that CK2 sites can modulate HOX function (Jaffe et al. 1997; Taghli-Lamalle et al. 2008), our results suggest that a single CK2 phosphorylation site is not sufficient to inhibit the limb repressive function of HOX proteins and multiple Ser/Thr phosphorylation sites may be required.

In this context, it is important to realize that a Hox protein can have a repressive effect on *Dll* and limb development without completely removing the limb appendage. It has been stated, for example, that UBX protein only evolved a limb repressive function late in insect evolution, and before that functioned only in modulating trunk appendage morphology (Palopoli and Patel 1998; Lewis et al. 2000). However, one way to modulate appendage morphology is to partially repress *Dll*, which appears to be a function of UBX in *Tribolium* development (Lewis et al. 2000). It has also been shown that ANTP is likely to modulate *Daphnia* limb morphology by repression of *Dll* in a few thoracic cells (Shiga et al. 2002).

In the context of *Artemia* embryos, limb development in the presence of UBX is probably due to a variety of evolved mechanisms, one component of which is the reduced repressive ability of the *Artemia* UBX protein on *Dll* transcription. It is possible that *Artemia* UBX protein is expressed at lower levels during limb bud development than in *Drosophila*, as UBX repressive function, at least in *Drosophila*, is highly sensitive to expression levels (Tour et al. 2005). It is also possible that the *Dll* limb *cis*-regulatory sequences have evolved lower affinities for UBX or its corepressors. In addition, *Drosophila Dll* can be repressed by UBX only at stages before the stage when *Dll* transcription is induced in limb primordia (Castelli-Gair and Akam 1995). This suggests that the exact timing of expression levels of HOX proteins with the potential to repress *Dll*, may be critical to limb number and morphology. Given that natural selection works with random genetic variations, it seems likely that evolutionary variations in many different molecules and processes in the limb development hierarchy have generated the great variation in limb shape and size found in different arthropods. In hexapod insects, this variation often reaches one extreme, that being the complete repression of *Dll* and limb development in the abdomen by UBX and ABD-A.

## Acknowledgments

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the on-line version of this article:

**Fig. S1.** *FP6.87* and *ABD-A* protein expression in *Artemia*. Double stains using the *FP6.87* antibody plus

$\alpha$ -*Artemia* *ABD-A* in *Drosophila* embryos ubiquitously expressing *Artemia* *ABD-ACD* show a strong nuclear staining with  $\alpha$ -*Artemia* *ABD-A* (D), and a weaker signal from the *FP6.87* antibody (C) (compare to negative controls in A, B) suggesting that the  $\alpha$ -*Artemia* *ABD-A* antibody may be a more sensitive antibody than *FP6.87* in fixed *Drosophila* embryos. In early stage *Artemia*, a smaller percentage of animals showed signal with the *FP6.87* antibody than with the  $\alpha$ -EN antibody. No detectable signal for *ABD-A* protein could be seen in any L1 to L4 stage animals (H, I; fluorescence in I is gut autofluorescence) for which *FP6.87* signal was detected (E, F). In the L12 stage *Artemia*, when all of the limbs in the trunk segments have finished differentiation, both *FP6.87* and *ABD-A* antibodies clearly detect protein in the same cells within the neuromeres (G, J and inset).

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