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RESEARCH ARTICLE

# Complex genetic interactions govern the temporal effects of *Antennapedia* on antenna-to-leg transformations in *Drosophila melanogaster*

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## Abstract

The putative regulatory relationships between *Antennapedia (Antp)*, *spalt major* (*salm*) and *homothorax (hth)* are tested with regard to the sensitive period of antenna-to-leg transformations. Although Antp expression repressed *hth* as predicted, contrary to expectations, *hth* did not show increased repression at higher Antp doses, whereas *salm*, a gene downstream of *hth*, did show such a dose response. Loss of *hth* allowed antenna-to-leg transformations but the relative timing of proximal–distal transformations was reversed, relative to transformations induced by ectopic Antp. Finally, overexpression of *hth* was only partially able to rescue transformations induced by ectopic Antp. These results indicate that there may be additional molecules involved in antenna/leg identity and that spatial, temporal and dosage relationships are more subtle than suspected and must be part of a robust understanding of molecular network behaviour involved in determining appendage identity in *Drosophila melanogaster*.

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## Introduction

Transformations from antenna-to-leg via mutations in genes such as *Antennapedia (Antp)* have provided insight into various aspects of homoeotic transformation and the relationship between embryonic and larval segment identity (Wakimoto *et al*. 1984). The distal-to-proximal temporal determination of regions during antenna-to-leg transformation (Postlethwait and Schneirerman 1969; Postlethwait and Schneiderman 1971; Gibson and Gehring 1988; Scanga *et al*. 1995) remains an intriguing and poorly understood phenomenon. Other studies have documented dynamic changes in dose response of individual structures during antenna-to-leg transformation (Larsen *et al*. 1996). While the analysis of the transcription activation pathway in which *Antp* participates has given us a paradigm for the circuitry of appendage identity (Bhojwani *et al*. 1997; Rieckhof *et al*. 1997; Duncan *et al*. 1998; Johnston*et al*. 1998; Kurant *et al*. 1998; Pai *et al*. 1998; Dong *et al*. 2000, 2001, 2002; Chu *et al*. 2002; Emerald *et al*. 2003; Suzanne *et al*. 2003), this work has not helped in the understanding of the dynamics of the sensitive period for the antenna-to-leg transformation.

Figure 1 shows the relationship between *Antp* and several genes involved with appendage determination, based on information in the literature. While this diagram clearly captures the findings that *Antp* is a negative regulator of *homothorax (hth)* (Casares and Mann 1998) which in turn positively regulates *spalt major (salm)* (Dong *et al*. 2002) and antennal fates in general (Casares and Mann 1998; Inbal *et al*. 2001), it does not capture spatial, temporal or quantitative aspects of the regulatory system which are crucial to under-

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standing developmental processes. This is in part due to the methods used to ascertain molecular relationships using genetic tools, such as, under and overexpressing gene products and associating these to changes in the expression of downstream genes. In the case of Antp and other transcriptional modulators, we know whether putatively interacting players are present in the same cells. However, other aspects of the molecular interactions have not been tested as carefully. In this study, we test some of the implications of the network (figure 1) to see if these relationships are consistent throughout development.

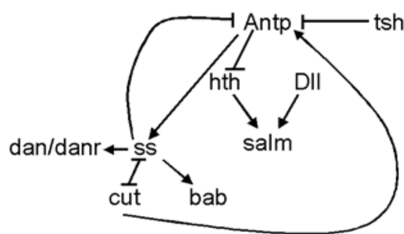


**Figure 1.** Summary of genetic interactions governing determination of antennal fates, based on previously reported observations as discussed in the text. *Antp* = *Antennapedia*, bab = *bric-a-brac*, dan(r) = *distal antenna (related)*, Dll = *Distal-less*, salm = *spalt major*, ss = *spineless*, tsh = *teashirt*.

If Antp is a negative regulator of *hth* and Hth is a positive regulator of *salm*, we expect that as the amount of Antp is increased in cells, this should result in a decrease of *hth* expression. Since Hth positively regulates *salm*, we would predict that Salm too would show reduced accumulations at higher doses of Antp. Further, since over–expression of *Antp* at different times in development has shown that antennal claw structures are produced before apical leg-like bristles, removal of Hth should produce transformations in the same time sequence. Finally, we would expect that ectopic expression of *hth* would rescue the antenna phenotype and reduce the homeotic transformation towards leg.

We present data to confirm that Antp represses the expression of *hth*, but *hth* expression does not show an inverse accumulation with increasing Antp expression. Nevertheless, *salm* demonstrates a dose responsiveness to Antp. Also, contrary to predictions, $hth^{-/-}$ clones did not show the same sequence of homeotic transformations as ectopic expression of Antp. Finally, *hth* over–expression produced only limited rescue of antennal structures in the presence of the ectopically overexpressing Antp allele, $Antp^{73b}$. We interpret these findings to suggest that when temporal and quantitative considerations are taken into account, the functional relationships between *Antp, hth* and *salm* are more complex than anticipated. We suspect that this may be a general finding in regulatory pathways, and suggest that deepening our perceptions of gene interactions will require incorporating spatial, temporal and quantitative relationships.

## Materials And Methods

***Stocks:***

HS-$hth^{15}$/*CyO*, HS-$hth^{3}$, $hth^{1422\text{–}4}$, $hth^{K1\text{–}8}$, $hth^{P2}$, and UAS-$hth^{12}$ courtesy of A. Salzberg. The three *hth* alleles described here are all *P* element insertions using a $P\{w^{+}, \mathrm{lacZ}\}$ (Kurant *et al.* 1998; Pai *et al.* 1998). The hypomorphic alleles were used both for generating mitotic clones as well as for visualizing *hth* expression. HS-*Antp* courtesy of M. Scott (Zeng *et al.* 1993). *y w* HS-*flp*22 ; FRT82 CD2 $My^{+}/TM2$, courtesy of G. Morata. All remaining stocks were obtained from the Bloomington Stock Centre: $ss^{a}$, $Antp^{73b}$/ *TM3 Sb*, $Antp^{Ns}$, *Dpp*-GAL4/*TM6B*, FRT 82 *Ubi*-GFP, FRT 82, *w*; $hth^{06762}$/*TM3 Sb*, $salm^{3602}$/*CyO*. Of the *P* element insertions into *hth*, only $hth^{1422\text{–}4}$ and $hth^{06762}$ reproduced expression patterns of *hth* transcripts.

***FLP-FRT system for generating somatic clones:***

The FLP-FRT system was used according to standard practices (Golic 1991; Xu and Rubin 1993). The $hth^{P2}$ and $hth^{1422\text{–}1424}$ mutations were recombined onto backgrounds with FRT82B. The *Y*/*y* HS-*flp*22; FRT82 *hth* / FRT82 CD2 $My^{+}$ genotypes were used for clonal analysis. Staged larval progeny were collected, and heat-shocked for six min at 37°C for one of several time points during development (24, 60, 68, 84, 96, 104 h after larva hatched respectively). Adults were collected after eclosion, and pharate adults were dissected out of pupal cases to score markers of antenna-to-leg transformation in marked clones.

***Mounting of antennae, legs and wings***

Prior to dissection, whole adult flies stored in ethanol were incubated in 1M *NaOH* at 65°C for 60 min to dissolve internal tissues, washed in 70% ethanol, and mounted in either Hoyer's or Faure's medium. Images were captured with a Hitachi KP-250 digital camera connected to an Olympus BX-60 microscope, using Image-Pro Software, or with a Zeiss Axio-Cam attached to a Zeiss Axio Scope 2, using Zeiss AxioVision (3.061).

***Sensitive period of ANTP-induced repression of hth expression***

We employed three genotypes for this assay; $w^{-}/w^{+}$; HS-*Antp*/$hth^{06762}$ was the primary treatment genotype, $hth^{06762}$/+ was employed to control for the marginal effects of heat shock on *hth* expression, and *w*; HS-*Antp*/+ was used as a positive control for analyzing phenotypic markers of homeotic transformation.

To study the temporal dynamics of the sensitive period of *hth* repression due to *Antp*, vials containing the genotypes described above were incubated at 37°C for 30 min to induce heat-shock-mediated expression of *Antp*. Based upon previous observations (Gibson and Gehring 1988; Scanga *et al.* 1995; Larsen *et al.* 1996), the following time points were used : 30, 43, 58, 64, 70, 76 after hatching (AH).

To study the dose-response relation, vials with timed larvae were incubated in 37°C water bath at 64 h AH for the following durations (based upon Larsen *et al.* (1996): 10, 20, 30 and 40 min.

Larvae of the genotypes $w^-/w^+$; HS-*Antp/hth*$^{06762}$ and $w^-/w^+$; HS-*Antp*/+ were also used for non-heat-shock-negative controls for monitoring changes in *hth* expression and phenotypic transformation. There was no evidence for any phenotypic transformations of antenna-to-leg for these controls.

Larvae of the $w^-/w^+$; HS-*Antp/hth*$^{06762}$ and *hth*$^{06762}$/+ genotypes were used for X-Gal staining of imaginal discs. Adults were also collected for phenotypic analysis of antenna-to-leg transformations. Prior to heat-shock, all vials were maintained at 25°C. At the appropriate time (see below), vials were incubated in a water bath at 37° ± 1°C.

Digital images of all of the stained imaginal discs were captured using a Hitachi KP-250 digital camera attached to an Olympus BX-60 microscope, using Image-Pro software systems. The diameter of the unstained central region of the antennal disc was measured, along with the diameter of the antennal disc to control for overall disc size (figure 3D). Remaining flies were raised to adults (including *w*; HS-*Antp*/+).

Similar results were observed in a pilot study using the *hth*$^{1422–1424}$ allele (results not shown). However, given that it demonstrated reduced viability, we did not use this allele further. Measurement error for this assay was assessed in a pilot experiment, and it explained ~ 3.5% of the total variation for spatial expression (data not shown).

***Sensitive period and dose-response for salm repression due to Antp***

Unless otherwise noted, the procedures used were identical to those discussed above for *hth*. Larvae of the genotype $w/+$; *salm*-lacZ/+; HS-*Antp*/+ were heat shocked as described above, at the following developmental times : 30, 44, 52, 62 and 72-AH. The dose-response analysis was performed at 62 h AH for the following durations : 15, 30, 45 and 60 min. Larvae were dissected at 96 - AH. Digital images for each stained disc were captured, and given a "dummy" label so that treatment levels would not be known. All images were scored on a scale of 0–2 (0 = no apparent expression) in a blind assay by a single individual (E.L.).

***Rescue experiments of the Antp$^{73b}$ phenotype using HS-hth***

The following genotypes were used for this experiment: *w*; HS-*hth*$^3$/+, *Antp*$^{73b}$/HS-*hth*$^3$ and *Antp*$^{73b}$/+. Larvae were collected, timed and heat shocked, as described earlier in this section. Unless otherwise noted, the duration of all heat shocks was 30 min. For the *w*; HS-*hth*$^3$/+, times for heat–shocks were : 52, 56, 60, 68, 72, 84 and 96-AH. This genotype was used to examine the effects of overexpression of *hth* on antenna, leg and wing development to determine if the phenotypic consequences of *hth* ectopic expression alone would make the rescue assay difficult to interpret. For the rescue assay, *Antp*$^{73b}$/HS-*hth*$^3$ and *Antp*$^{73b}$/+ (negative control) were heat shocked at the following times : 36, 48, 60 and 72-AH. This experiment was then repeated for several later times : 60, 68, 72, 76, 84 and 96-AH.

***Collection and staging of timed first instar larvae***

Newly hatched first instar larvae were collected (see Dworkin 2005, for details) on apple juice - agar media supplemented with yeast paste. Larvae were transferred to regular food vials at constant density (30 larvae per vial).

***Antibody staining of imaginal discs***

Tissues were fixed with 4% paraformaldehyde in PBS, for 20 min at room temperature (RT) in 24-well cell-culture plates, followed by a 3 min postfixation in methanol. Tissues were rinsed 3 times and washed 3 times for 20 min each in PBT, blocked overnight in PBTBS (PBT + 0.1% bovine serum albumin + 2% goat serum) at 4°C. Following blocking, the tissue was incubated with the primary antibody (concentrations specified for each experiment) in PBTBS overnight at 4°C. Tissues were then rinsed and washed in PBT 3 times for 20 min each. Tissues were reblocked as before. Secondary antibody was applied in PBTBS and incubated overnight at 4°C. The tissue was then washed in PBT 3 times for 20 min each. For secondary antibodies conjugated to horseradish peroxidase, tissues were incubated (in the dark) in DAB–PBT solution (stock solution of 1 mg/ml, diluted 1 : 4 with PBT) for 10 min. Following this incubation, 0.5 ml of 30% $H_2O_2$/500 $\mu$l of DAB-PBT was added, and tissue was monitored until stained. Tissues were then washed several times in PBS, and imaginal discs were mounted in glycerol. $\alpha$-ANTP (used at 1 : 100 dilution, courtesy of Dr. T. Kauffman). $\alpha$-EXD, courtesy of Dr. R. Mann (Rieckhof *et al.* 1997) were used at 1 : 400. Secondary antibodies were used at 1 : 500 dilution ($\alpha$-mouse-HRP and $\alpha$-rat-HRP, Jackson Labs).

***X-Gal staining of imaginal discs***

To monitor gene expression using lacZ enhancer traps, X-Gal staining was employed (based on procedure from D. Godt). Larval tissue from timed larvae was fixed in 1% glutaraldehyde in PBS, for 5–7 min at RT. The tissue was then rinsed 2 times in PBT (0.1% Triton-X100 in PBS) and washed in PBT 2 times for 15 min each at RT. The tissue was incubated in a solution of X-Gal staining buffer containing 1 : 50 dilution of X-Gal (8% in DMSO) at 37°C for one hour (*hth*-lacZ) or two hours (*salm*-lacZ). The tissue was rinsed, washed (PBT) and mounted in 70% glycerol in PBS. For each enhancer trap, all samples were processed simultaneously, using common solutions. Unless otherwise noted, timed larvae were dissected at 96 h AH.

***ANTP half-life assay***

Timed HS-*Antp* homozygous larvae were heat–shocked at 37 ± 0.5°C. The timing of the heatshocks were set so that

larvae could be dissected at 96 h AH. The treatments were (time for ANTP to breakdown given in paranthesis): (a) no heat-shock (control), (b) 72 h AH (24 h), c) 80 h AH (16 h), (d) 88 h AH (8 h), and (e) 96 h AH (0 h). All heatshocks were of 40 min duration at 37°C.

All steps of the $\alpha$-ANTP antibody staining procedure were carefully controlled so that all treatments were handled identically with respect to staining procedures. All images were digitally captured under identical light conditions. Relative abundance of Antp was measured using Scion-Image software (Scion Corporation). The antennal region of the disc in the image was digitally isolated and the average density of staining was recorded. For each picture, two representative samples of background (empty space on the slide, recorded in the picture) were measured, averaged and subtracted from the staining density of the antennal disc.

***Statistical analysis***

ANTP half-life experiment : Since the expression data were not normally distributed, both parametric analysis of variance (ANOVA) and a nonparametric alternative, the Kruskal–Wallis test (Proc NPAR1WAY in SAS) were employed, fitting the model

$$Y_{ij} = \mu + T_i + \epsilon_{ij}$$

For the linear regression, Proc REG (SAS) was used for fitting the function, to estimate the half-life. An analysis of repeatability suggests that measurement error is less than 6.5% of total variation for Antp staining in the discs (data not shown).

***Sensitive period of ANTP-induced repression of hth expression :***

To examine the effects of ectopic expression of Antp on *hth* expression, the following general linear model was fit in Proc GLM (SAS):

$$Y_{ijk} = \mu + T_i + G_j + T \times G_{ij} + C_k + \epsilon_k,$$

In this model, $Y_{ijk}$ is the raw value for relative *hth* repression, $\mu$ is the grand mean, $T_i$ is the effect of heat-shock at time $i$, $G_j$ is the genotypic effect for genotype $j$, $e$ is the term error representing residual individual error, and the covariate $C_k$, is the diameter of the antennal disc for this analysis of covariance (ANCOVA). Including overall disc size as a covariate controls for any size dependent effects. To test for the effects of ectopic Antp at individual time points, we used both Dunnett's test, and one-tailed $t$-tests (corrected for multiple comparisons, using sequential Bonferroni correction) relative to the control lines. For dose response, the same model was used, but $T_i$ represents the heat-shock of length $i$, in a linear regression.

This same framework was used in the context of the dichotomous response variables, utilizing a logistic regression model (**??**) or logit model (**??**). Otherwise, the model structure is analogous. Proc LOGISTIC and Proc CATMOD (SAS) were utilized, which derive maximum-likelihood estimates for parameters, and for which likelihood-ratio tests can be used to assess the significance of the models (**?**).

## Results

***Breakdown of ANTP over time; antennal disc stainings***

To improve the interpretation of the temporal effects of ectopic Antp on *hth* expression, it seemed logical to examine the stability of the Antp protein in the antennal discs. Earlier studies (Gibson and Gehring 1988; Scanga *et al.* 1995), suggested that the protein was undetectable between 4 and 8 h after ectopic expression. However, in a pilot study, no significant reduction in relative staining was observed in this time period. Therefore we extended this analysis to allow for up to 24 h for the protein to breakdown.

Discs stained right after induction of ANTP show the highest levels of staining, and this level decreases progressively over time (figure 2). Interestingly, the staining intensity does not substantially decrease between 0 and 8 h after induction, but in the next 8 h interval, there is a significant decrease in intensity. By 24 h after induction, ANTP staining is indistinguishable from the controls. Using the nonparametric Kruskal-Wallis test (**?**), a significant effect of time for breakdown ($\chi^2$ = 147.0, df = 4, $P < 0.0001$) was observed. To determine if each time point was significantly different from the control, a one-tailed Dunnett test was employed. All time points, except the 24 h after heat-shock treatment, yielded significantly greater staining than the control (figure 2). Using Tukey's HSD test for multiple comparisons, it was also demonstrated that there was no difference in relative intensity of staining between 0 and 8 h after induction of Antp (data not shown). The linear estimates for the protein half-life of Antp is 12.01 h. These quantitative estimates suggest that the earlier results were likely underestimates, possibly due to the qualitative comparisons performed. If presence of Antp is required to maintain the repression of *hth*, then a conservative estimate suggests that it can do so for at least 24 h after ANTP induction.

***Temporal dynamics of Antp expression on hth***

The antennal disc possesses an extremely dynamic pattern of transformation towards leg fate based upon both timing and dose of ectopic expression of Antp (Gibson and Gehring 1988; Scanga *et al.* 1995; Larsen *et al.* 1996). Thus we predicted that *hth* repression via Antp should be equally dynamic. However, a single short pulse of ectopic expression of *Antp* in the antenna disc results in only partial transformation, depending on the timing and dose (Gibson and Gehring 1988; Scanga *et al.* 1995). Thus, it was unclear if strong qualitative effects on *hth*-driven lacZ expression would be observed; such as those seen with the neomorphic allele $Antp^{73b}$

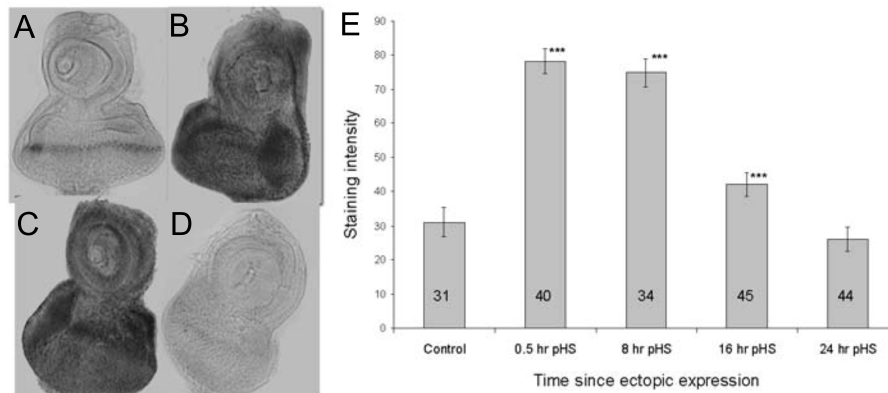


**Figure 2.** Antp breakdown with time. (A) Non heat-shock control showing no expression in the antennal disc. (B) 30 minutes after heat shock, most staining is nuclear. (C) 8 hours after heat shock, there is considerable staining, though it has diffused somewhat. (D) 24 hours after heat shock, there is virtually no Antp found in the antennal disc. (E) Graph demonstrating the quantitative changes in the levels of Antp after the initial pulse. All time points, expect 24 hours post-heatshock (pHS) are significantly different from the nonheatshock controls (*** $p < 0.0001$, one-tailed Dunnett's test).

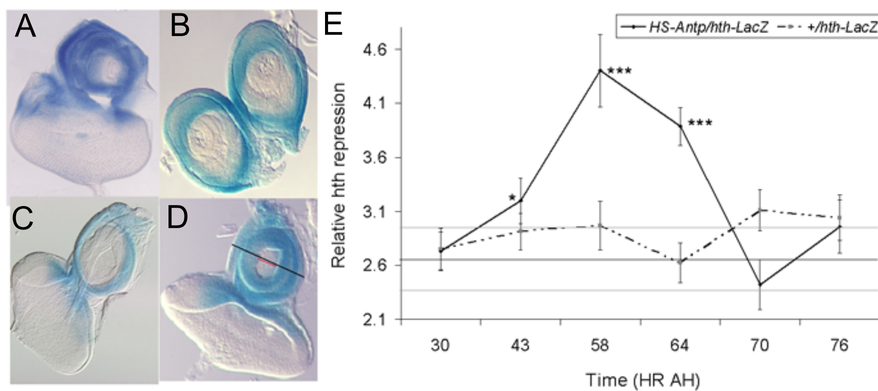


**Figure 3.** Antp represses *hth* in a time dependent manner; *hth* expression monitored using the $hth^{06762}$ enhancer trap. (A) Expression of *hth* in wild-type late third instar antennal imaginal disc. (B) Expression of *hth* in wild-type late third instar leg discs, demonstrating the restriction of expression to the presumptive proximal leg. (C) Expression of *hth* in an $Antp^{73b}$ antennal disc, mimicking the expression in the leg disc. (D) The measures used for quantitatively monitoring *hth* expression. The diameter of the unstained region (red) was measured, as was the diameter of the antennal disc (black) to control for overall growth. (E) Graphical illustration of the repression of *hth* due to a short pulse of Antp from a HS-*Antp* transgene. *hth* expression was significantly repressed relative to the controls at three time points (* $P < 0.05$, *** $P < 0.0001$) during late second and early third instar. The horizontal black line (gray lines = 95% CI) represents nominal expression observed in flies where *Antp* was not ectopically expressed. The three time points were significant using both one-tailed *t*-test (HS-$Antp/hth^{06762}$ *versus* $hth^{06762}/+$) and one-tailed Dunnett's tests (HS-$Antp/hth^{06762}$, heat-shocked control *versus* nonheat-shocked controls). Sample sizes (treatment/control) as follows (time point 30: 38/38, 43: 30/43, 58: 11/23, 64: 38/37, 70: 24/34, 76: 21/27, non-heat shocked control 26).

(figure 3c). Therefore, a quantitative measure of *hth* repression was devised based upon changes in *hth* expression in the presumptive distal region, relative to the rest of the disc using a *hth* enhancer trap line (figure 3d).

The quantitative pattern of repression of *hth*-lacZ by Antp is consistent with a sensitive period for *hth* repression (figure 3e). Early in the second instar of larval development (30 h AH), there is no evidence for an expression difference. However, during late second and early third instar (43, 58, 64 h AH) there is a relative restriction in the domain of expression of *hth*-LacZ. By mid-third instar, this difference seems to disappear, consistent with morphological and other

molecular markers of homoeotic transformation (Scanga *et al.* 1995; Larsen *et al.* 1996). While the results of the overall ANCOVA suggest a strong interaction between time and genotype ($F_{5,276} = 26.6$, $P < 0.0001$), of particular interest is the effect of HS-*Antp* on *hth* expression at specific periods of time during the sensitive period, relative to the controls. We first approached this question by using independent contrasts between control–treatment pairs. For each time point, contrasts using one-way ANOVA's (figure 3e), corrected for multiple contrasts, suggest that the three central time points are all significantly different than their respective controls. Similar results were observed using one-tailed Dunnett's test (controls for experimentwise type I error) of the least-square estimates at each time point compared with the non-heat-shocked control (figure 3e). The sensitive period of HS-*Antp* induced repression of *hth*-lacZ is in agreement with previous observations for morphological markers of homoeotic transformation (Gibson and Gehring 1988; Scanga *et al.* 1995; Larsen *et al.* 1996) (see figure 5), consistent with the hypothesis of Antp-induced homoeotic transformation acting through repression of *hth* (Casares and Mann 1998).

Previous work has demonstrated that the dose-response relationship between ectopic expression of Antp and homoeotic transformation is complex with respect to the temporal conditions of misexpression (Larsen *et al.* 1996). In particular, transformation towards apical bristle shows a positive dose-response at around 64 h AH, while 4 h earlier the relationship is negative (Larsen *et al.* 1996). Consistent with previous results, transformations to bracted leg bristles ($F = 99.4$, df = 1, $P < 0.0001$) and apical bristle (df = 1, likelihood ratio = 7.7, $P = 0.006$) both show a positive dose-response relationship with duration of heat shock. Surprisingly, there was no evidence for dose-dependent *hth* repression due to Antp at this time point ($F = 0.06$, df = 1, $P = 0.8$). It is unclear why these results differ from those observed for the sensitive period, but may be due to complete repression of *hth* by low levels of Antp.

In addition to monitoring *hth* expression, we also monitored the effects of HS-*Antp* on expression of *salm*-LacZ. *salm* is expressed in the antennal, but not in the leg imaginal disc, is down-regulated in the presence of Antp (Wagner-Bernholz *et al.* 1991), and is a positively regulated target of Hth (Dong *et al.* 2002). Thus *salm* should show a negative dose-response relation with Antp. Unlike the results with *hth* expression, there appears to be a simple nonlinear negative relationship between dose of Antp and *salm*-LacZ expression (figure 4). Analysis using a logistic regression framework is consistent with a significant effect of dose of Antp on *salm* repression (slope = $-0.12$, df = 1, likelihood ratio = 96.4, $P(\chi 2) < 0.0001$). Thus, the results of morphological markers of transformation along with *salm* expression are consistent with a dose-dependent effect of Antp, while the effects of *hth* are not.

#### *The temporal dynamic of antenna-to-leg transformation due to ectopic expression of Antp differs from loss of Hth*

If the homoeotic transformations induced by ectopic expression of *Antp* are a direct result of repression of *hth*, then it would be expected that the temporal sequence of homoeotic transformation observed in a HS-*Antp* background should be mimicked in Hth loss of function clones in the antenna. Recombinant FRT chromosomes bearing a strong hypomorphic allele *($hth^{P2}$)* were generated so as to facilitate the study of loss of function of Hth. Given that this work was done in a *Minute* background, which retards the rate at which development proceeds, only qualitative comparisons could be made with the effects of HS-*Antp*. Further, the nature of mitotic clone generation allows only for the comparison of the end of sensitive periods. Given these caveats, we still expect that antenna-to-leg transformations occur in a distal to proximal sequence, as with HS-*Antp*. Specifically, we predict that the sensitive periods for the claw, the most distal marker, should end earlier than the sensitive period for the more medial marker of transformation, the apical bristle.

As shown in figure 5, the sensitive period for transformation to claw ends later than that for apical bristle in the loss of function *hth* clones (figure 5f). This is reverse of the predicted order, and that observed for HS-*Antp* in this study (figure 5e) and previously (Gibson and Gehring 1988; Scanga *et al.* 1995). It is worth noting that the general marker of antenna-to-leg homoeotic transformation, the presence of bracted bristles, shows, as expected, a long sensitive period suggesting that the assay itself has been effective in inducing transformation, and that the temporal results with *hth*-lacZ expression are meaningful.

#### *The temporal dynamics of rescue: turning antennae transformed towards leg back to antennae*

Under the hypothesis that loss of *hth* mediates the effects of Antp induced antenna-to-leg transformations, we predicted that ectopic expression of *hth* should rescue the *Antp* homoeotic phenotype, possibly in a time-dependent manner. (Yao *et al.* 1999) overexpressed *hth* using a *Dpp*-Gal4 driver in an $Antp^{Ns}$ background and interpreted their results as demonstrating that this was sufficient to rescue the antenna-to-leg transformation phenotype. However, overexpression of *hth* leads to ablation of distal antennal and leg structures (figure 3 of Yao *et al.* 1999, personal observation). Given that loss of *hth* causes distal antenna-to-leg transformation, the phenotypic effect of overexpression using Gal4 drivers may compromise any interpretation.

As an alternative strategy, we utilized a HS-*hth* line in the background of a strong neo-morphic allele, $Antp^{73b}$. This allows for control of the dose of Hth, as well as timing of expression. Given the results of overexpression discussed above, we were also interested in the sensitive periods for

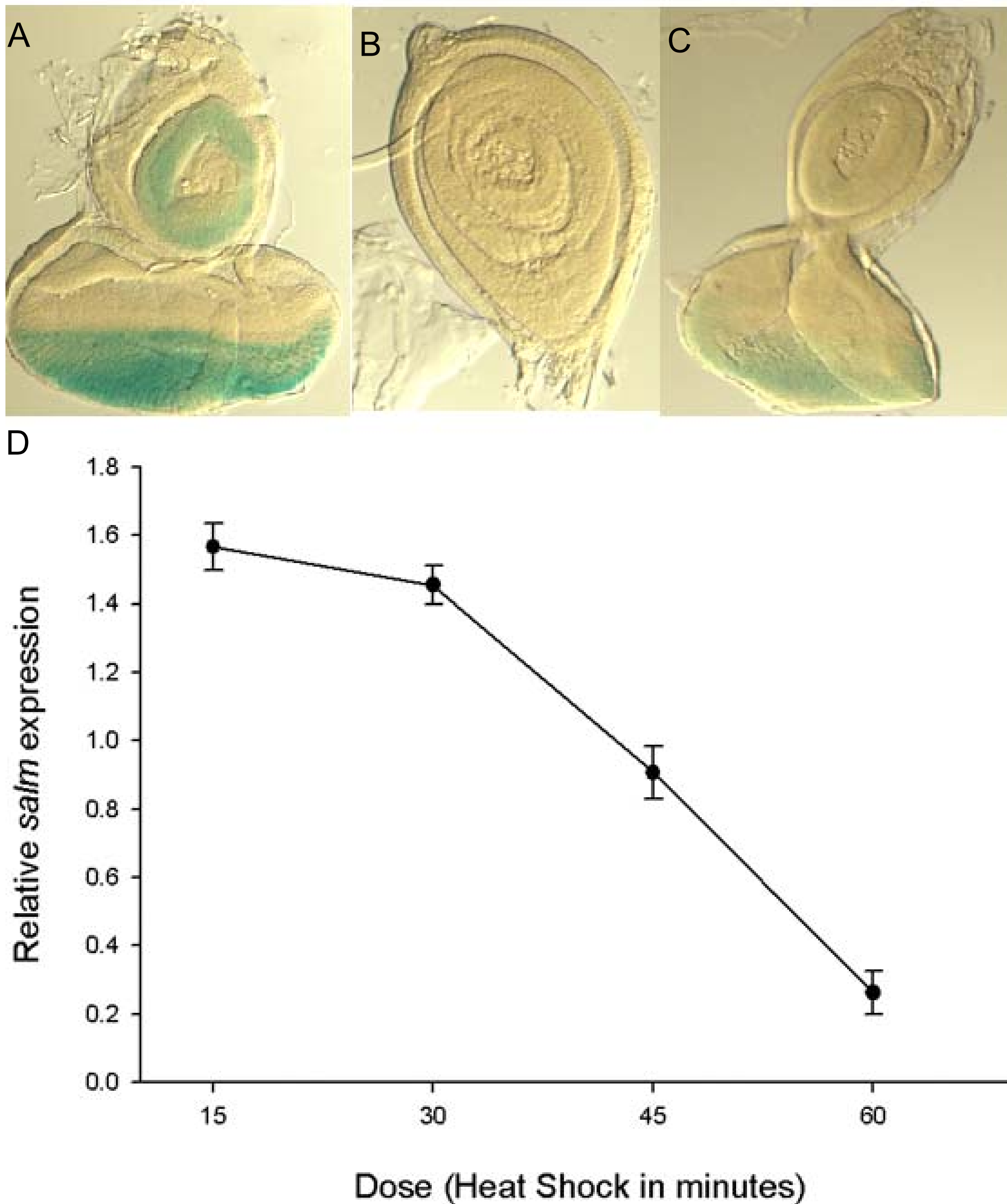


**Figure 4.** *salm* demonstrates a dose-response to ectopic expression of *Antp*. (A) Expression of *salm* in a third instar antennal disc as monitored using the *salm*-LacZ enhancer trap ($salm^{3602}$). (B) No evidence for *salm* expression in the leg imaginal disc, nor in the genetic background of $Antp^{73b}$ (C). (D) Quantitative demonstration of decreasing *salm* expression with increase in ectopic *Antp*.

phenotypes induced by ectopic Hth, as this will reduce misinterpretation due to confounding effects from the ablation of distal structures. Therefore in parallel to the $Antp^{73b}$ rescue experiment, we examined the effects of HS-*hth* by itself on legs, antennae, and wing structures. To determine whether a short pulse of HS-*hth* is sufficient to show biological activity, we examined the localization of Exd protein, which is only localized to the nucleus in combination with Hth. After induction of Hth, Exd was localized to the nucleus in all imaginal discs examined (data not shown), consistent with

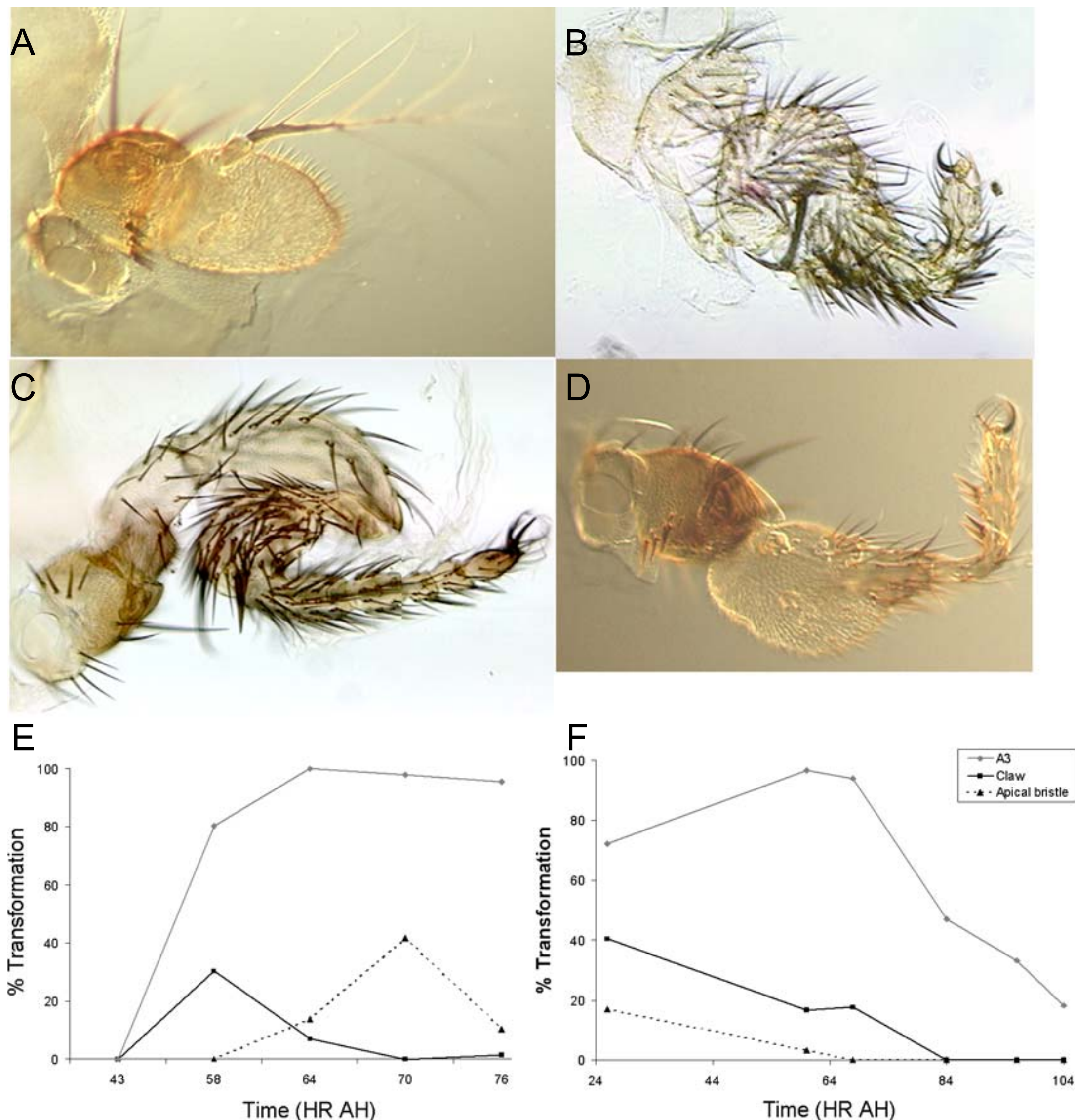


**Figure 5.** Sensitive periods for antenna-to-leg transformation. (A) wild-type antenna. (B) $hth^{P2/P2}$ clone in the antenna, demonstrating a partial transformation towards leg fates. (C) Ectopic expression of *Antp* in the antennal disc can result in an almost complete transformation as in this $Antp^{73b}$ genetic background. (D) A short pulse of ectopic *Antp* using a *HS-Antp* transgene during early third instar results in only a partial transformation of distal structures. (E and F) Graphical summary of the sensitive periods for antenna-to-leg transformations due to ectopic expression of *Antp* (E) or loss of *hth* (F). While transformation towards leg fate shows a distal to proximal temporal sequence due to ectopic expression of *Antp*, the opposite is observed in the $hth^{-/-}$-clones. A3 represents the presence of leg-like bristles on the third antennal segment, a general marker for transformation to leg.

previously published observations (Rieckhof *et al*. 1997; Kurant *et al*. 1998; Jaw *et al*. 2000). Thus a short heat shock is sufficient to induce the activity of Hth.

***The sensitive period of antenna and leg to overexpression of Hth***

While overexpression of *hth* using a *Dpp*-Gal4 driver causes loss of distal structures in the antenna, the short pulse of HS-*hth* was not sufficient to induce this phenotype in the antenna at any time point tested. However, the effects on leg development were severe (figure 6), and demonstrate interesting patterns of temporal sensitivity (figure 7). A number of morphological markers on the leg showed definite sensitivity to ectopic Hth throughout the leg disc, such as the apical bristle on the second leg, and the presence of ectopic sex comb teeth on the fifth tarsal segment of the first leg (figure 6).

In general, the leg seems sensitive to ectopic Hth between 60 and 70 h AH, corresponding to the early third instar (figure 7). The sensitive period for segmental fusions in the leg appears to last somewhat later. Unlike what has been observed for antenna-to-leg transformation, there is no

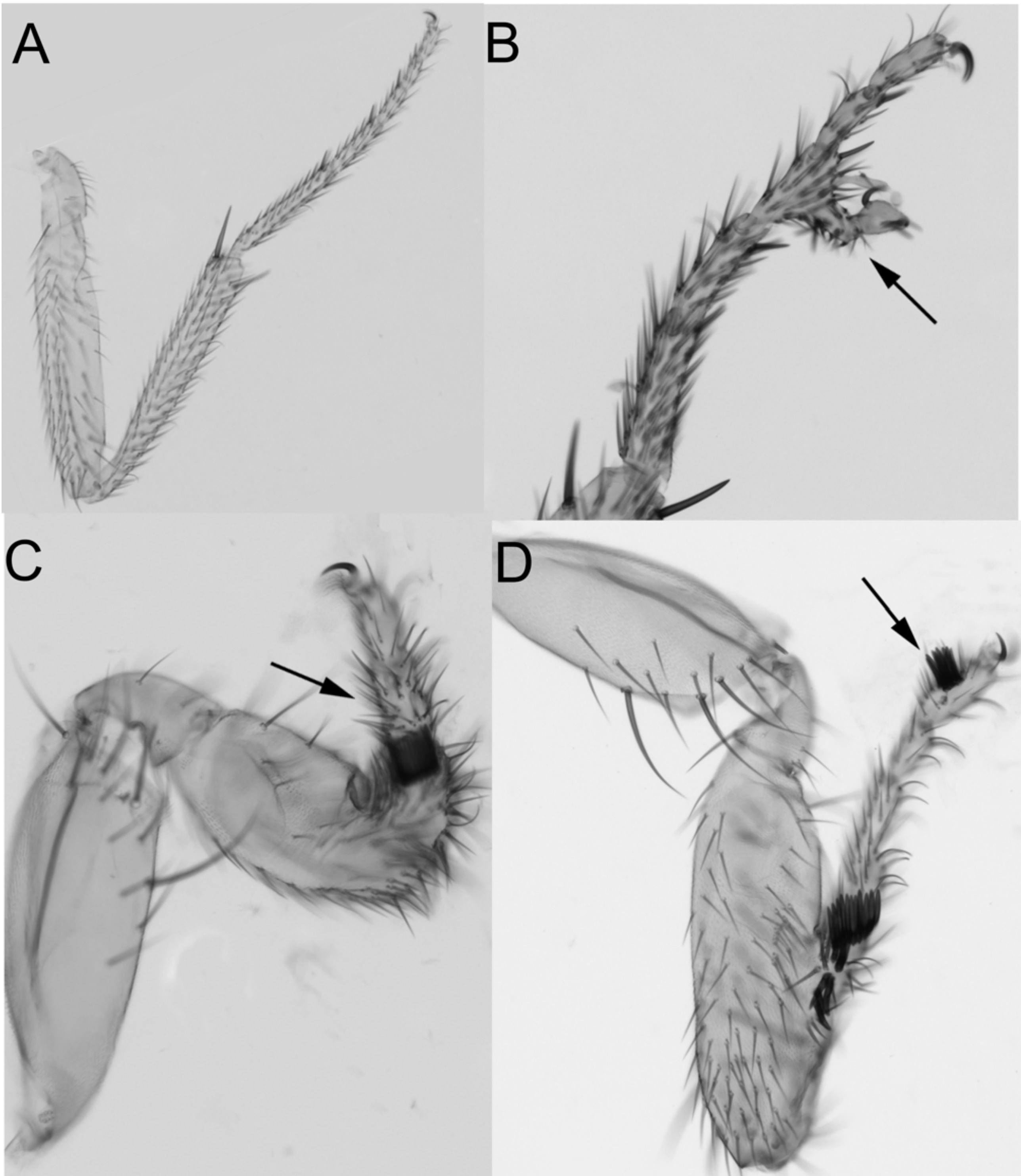


**Figure 6.** Phenotypic effects of ectopic *Hth*. (A) Wild-type second leg at 40X. (B) Tarsal bifurcation on second leg in a HS-$hth^3$/+ leg heat shocked at 52 hours after hatch (HRAH), 100X. (C) Pro-thoracic leg showing fusion of distal segments, and a failure of the sex comb to rotate in a HS-$hth^3$/+ leg heat shocked at 60 hours HRAH, 100X. (D) Ectopic sex comb teeth on the most distal tarsal (T5) segment (arrow) in a HS-$hth^3$/+ individual heat shocked at 56 HRAH, 100X.

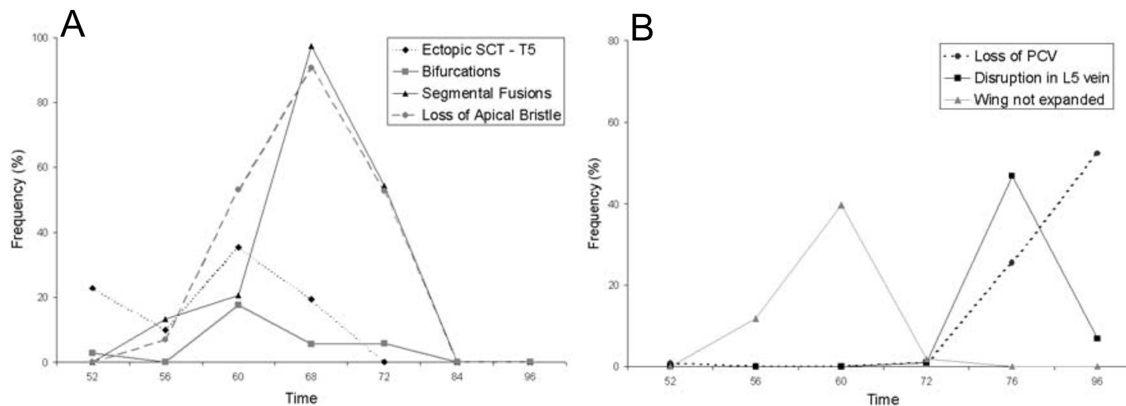


**Figure 7.** Sensitive period of legs and wings to ectopic *Hth*. (A) Frequency of abnormal leg phenotypes. Data taken for the pro-thoracic leg, except for the loss of the apical bristle (second leg). Similar results were observed for segmentation and bifurcation phenotypes on the second leg. A minimum of 20 legs per time point were examined for each leg. SCT on T5 = sex comb teeth on fifth tarsal segment. (B) Sensitive period for the wing to ectopic *Hth*. PCV = posterior cross vein. L5 = fifth longitudinal vein. A minimum of 50 wings per time point were examined.

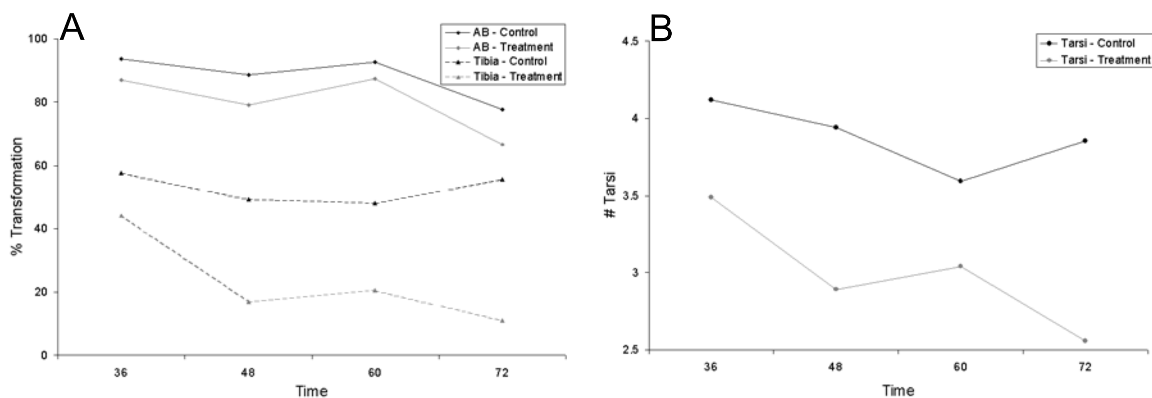


**Figure 8.** No sensitive period for rescue of the $Antp^{73b}$ phenotype by HS-*hth*. (A) While ectopic expression of *Hth* decreased the frequency of transformation towards both apical bristle (AB) and tibia in $Antp^{73b}$ flies, there was no evidence for a specific period of rescue. (B) Similar observations were made for number of tarsal segments on the transformed antenna. A minimum of 50 antennae were examined per time point.

evidence for a distal to proximal wave of sensitivity in the leg, suggesting that this tissue sensitivity may be unique to the antennal disc. However the leg does appear to lose sensitivity to ectopic Hth at approximately the same developmental time as the antennal disc loses its competence to transform towards leg identity. One surprising finding is that there was no evidence for the short pulse of ectopic Hth causing a loss of bracted bristles on the distal leg (Azpiazu and Morata 2002). Interestingly, the loss of the posterior cross-vein (PCV) or the L5 vein did not appear at high frequency until beyond 70 h AH (figure 7b), demonstrating tissue and trait-specific sensitivities to the effects of ectopic Hth.

Perhaps the most peculiar phenotype observed was the ectopic formation of sex comb teeth on the most distal tarsal segment (T5), with none on the more proximal tarsal segments (figure 6d). This phenotype is most interesting since it has not been observed in mutants which alter the positioning of the sex comb teeth, and is unlike patterns observed in other drosophilid species (Kopp and True 2002; Dworkin 2005). The sensitive period for this phenotype is similar to the formation of ectopic sex comb teeth near the native site (figure 7a), but shows no distal to proximal wave of sensitivity.

As shown in figure 8, the qualitative pattern suggests that HS-*hth* is sufficient to induce partial rescue of the antenna-to-leg transformation relative to the $Antp^{73b}$ controls. Thus, the evidence is consistent with rescue of the antenna-to-leg phenotype by HS-*hth*. Surprisingly, we found no consistent evidence to suggest that there was a sensitive period for the rescue (figure 8). This may be due to the fact that the sensitive period for the majority of the leg defects strongly overlaps with that of the sensitive period for antenna-to-leg transformations. Thus, these results make interpretations of rescue during this period (∼ 60 – 72 h AH) difficult. Thus, we ar-

gue that interpretation of rescue of the *Antp*$^{73b}$ phenotype by ectopic expression of *hth* should be made with caution.

## Discussion

Determining the nature of interactions between genes has been a major goal of developmental genetics, and in *Drosophila* there is high resolution of many genetic pathways involved with a variety of developmental processes (reviewed in Held 2002). Nevertheless, most studies have examined such interactions as a single snap-shot of development, and extrapolate such findings to the remainder of the ontogeny. It is clear from previous studies that the function(s) of genes may change during the course of development (Steinbach *et al*. 1997; Roch and Akam 2000). In this study, we explored the temporal dynamics of some of the canonical genes involved with fate determination between antennal and leg fates in *Drosophila*. Earlier work demonstrated that the antenna-to-leg homoeotic transformation caused by ectopic expression of *Antp* was both stage (Gibson and Gehring 1988; Scanga *et al*. 1995) and dose dependent (Larsen *et al*. 1996). Recent work has suggested that Antp causes the transformation by repressing *hth* in the antennal disc, which regulates tissue fates (Casares and Mann 1998). We tested this hypothesis by examining whether *hth* is repressed by Antp in a stage-dependent manner. While repression of *hth* does correspond to the known sensitive period of the antennal disc to Antp, there are a number of results inconsistent with the strict interpretation of *hth* mediating all of the Antp induced effects. First *hth* repression did not show dose-dependent response to Antp, while morphological markers of transformation and *salm* expression did. Second, the distal to proximal wave of homoeotic transformation observed for morphological markers due to ectopic expression of *Antp* is not observed in loss of function *hth* clones in the antenna. Finally, while ectopic expression of *hth* in an *Antp*$^{73b}$ background does appear to rescue the homoeotic transformation, there is no evidence for a sensitive period for the rescue. In summation, these results are consistent with *hth* mediating some of the effects of Antp in the antennal disc. However, it is necessary to hypothesize that Antp has additional target genes which respond at other times during the sensitive period. The results of the dose-response experiment to HS-*Antp* suggest that perhaps *hth* is repressed by low concentrations of Antp protein, and that other targets are repressed at higher doses.

What other candidates might *Antp* be regulating independent of *hth*? While there are several candidate genes whose loss of function phenotype results in antenna-to-leg transformation such as *Dll, ss, babII* and *dan/danr*, no direct connection has been made. Over expression of both *ss* and *babII* using *Dpp*-Gal4 in an *Antp*$^{73b}$ background can rescue the antenna-to-leg phenotype (data not shown). It is interesting that in one context (*E132*-Gal4 UAS-*babII*) ectopic expression of *babII* causes antenna-to-leg transformations (Cabrera *et al*. 2002), while in another (UAS-*babII*/*Dpp*-Gal4 *Antp*$^{73b}$) it can rescue such transformations, arguing for complex regulation of these tissues. Evidence suggests that *ss* acts downstream of *hth*, and thus may be an indirect target of the effects of Antp (Dong *et al*. 2002). However, preliminary evidence from the quantitative assay in the current study suggest that the *hth* expression domain is restricted in a *ss* mutant background (personal observation). As reviewed recently (Chu *et al*. 2002; Dong *et al*. 2002; Emerald *et al*. 2003; Suzanne *et al*. 2003; Emerald and Cohen 2004) there are a number of genes involved with the antennal *versus* leg determination, and many subtle interacting effects are likely to still be resolved. We believe that resolving the temporal dynamics of these interactions is likely to be an important step in this understanding.

#### *Temporal sensitivity of antenna-to-leg transformation versus proximal–distal axis specification*

The sensitive periods of the antenna and leg discs show some differences, and in our study we did not observe a distal to proximal wave with respect to the affects of ectopic Hth. Nevertheless, in a more general sense, the sensitive periods for both the antenna and leg discs appear to begin and end at approximately the same points during ontogeny. One surprise finding was that overexpression of *hth* was not sufficient to repress bracts on the distal leg, at least during early larval development. Previous work has shown that bracts have a sensitive period later during pupal development (Held 1990, 2002), and the appearance of bracted bristles on antennae in HS-*Antp* flies is generally uncoupled from other aspects of appendage identity (Scanga *et al*. 1995). Thus, this suggests that not all aspects of the proximal–distal axis are specified during early development.

#### *Towards a mechanism for tissue competence with respect to regulation by Antp*

The results presented in this study suggest that at least part of the homoeotic transformation due to ectopic Antp is mediated through repression of *hth* in the antenna. What is yet unclear is why *hth* expression itself, along with certain morphological markers of antenna-to-leg transformations, shows a sensitive period for competence of transformation. Is it due to the complex interplay of the gene products that are themselves involved with appendage identity? Or, is there a mechanism that controls tissue competence that interacts with the genes? A previously suggested possibility is that the period of competence to Antp is mediated by changes in chromatin structure (Larsen *et al*. 1996). In *Xenopus*, the amount of somatic Histone H1 variant can alter the period of competence for neural identity (Steinbach *et al*. 1997). More effort is needed to test this possibility, which will be complicated by the fact that particular sensitivities may occur in very few cells at restricted times and gene doses.

***Evolutionary implications***

While such temporal and dose sensitivity is curious with respect to the antenna-to-leg transformation system, perhaps it is not surprising. A number of recent studies point to a diverse set of mechanisms regulating antennal versus leg fates in arthropods. For instance, *Dll* function is conserved along the proximal–distal axis of ventral appendages, yet its regulation of antennal *versus* leg fates does not appear to be conserved in the milkweed bug *Oncopeltus* (Angelini and Kaufman 2004; Herke *et al*. 2005), nor in the beetle *Tribolium* (Beermann *et al*. 2001). The pathways leading to appendage identity appear to demonstrate a high degree of flexibility, relative to the function of genes involved with specifying proximal–distal fates (Panganiban *et al*. 1994, 1997; Beermann *et al*. 2001; Beermann and Schroder 2004). Thus, the appendage determination pathway may provide an excellent model for the study of the evolution of genetic interactions, and will hopefully be explored in more detail in a number of different nonmodel organisms.

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