LETTER: DISCOVERIES

Cryptic variation in quantitative spatial gene expression in *Drosophila*

Sammi Ali[1*], Sarah A. Signor[1*+], Konstantin Kozlov[2], Sergey V. Nuzhdin[1,2]

[1]Section of Molecular and Computational Biology, University of Southern California, Los Angeles, California, 90089 USA

[2]Department of Applied Mathematics, St. Petersburg State Polytechnic University, St. Petersburg, 195251, Russia

*Co-first author

[+]Corresponding author: 1050 Childs Way, RRI Hall,
Molecular and Computational Biology
University of Southern California
Los Angeles, 90089
ssignor@usc.edu
Phone 213-740-3065

ABSTRACT (150 words)

Robustness in development allows for the accumulation of cryptic variation, and this largely neutral variation is potentially important for both evolution and complex disease phenotypes. However, it has generally only been investigated as variation in the response to large genetic perturbations. Here we use newly developed methods to quantify spatial gene expression patterns during development of the *Drosophila* eye disc, and uncover cryptic variation in wildtype developmental systems. We focus on four conserved morphogens, *hairy*, *atonal*, *hedgehog*, and *Delta*, that are involved in specifying ommatidia in the developing eye. We find abundant cryptic variation within and between species, genotypes, and sexes, as well as cryptic variation in the regulatory logic between *atonal* and *hairy* and their regulators, *Delta* and *hedgehog*. This work paves the way for a synthesis between population and quantitative genetic approaches with that of developmental biology.

36 **RESULTS AND DISCUSSION (2,000 words)**

37 Cryptic variation accumulates due to the robustness of developmental systems to mutational
38 perturbation (Duveau and Felix 2012; Felix 2012; Felix and Barkoulas 2012; Kienle and Sommer
39 2013; Paaby and Rockman 2014; Paaby and Gibson 2016). Although the final phenotype is the same,
40 the presence of this cryptic variation effects the response to mutational or environmental perturbation,
41 and thus the evolutionary potential of the phenotype. In the past cryptic variation has been
42 experimentally exposed through the use of synthesized genetic backgrounds with large perturbations
43 that prevent buffering of variation (Braendle et al. 2010; Woodruff et al. 2010; Duveau and Felix 2012;
44 Chandler et al. 2014; Taylor and Ehrenreich 2015). While this work provides valuable insights into the
45 importance of cryptic variation, cryptic variation in developmental processes in natural genotypes has
46 not been experimentally observed.

47 Indeed, natural genetic variation within populations has long been the purview of evolutionary and
48 population geneticists, while developmental biologists focus on the effect of large mutations in
49 otherwise isogenic backgrounds (Paaby and Gibson 2016). This dearth of work on cryptic
50 developmental variation in wildtype genetic backgrounds is in part because developmental
51 approaches have long been restricted to data that is at best semi-quantitative (i.e. *in situ* hybridization,
52 antibody staining). Indeed, gene expression studies are generally spatial or quantitative, but not both.
53 Without quantitative replication, there can be no rigorous statistical testing when developmental
54 processes are compared among conditions, including health versus disease. This is especially
55 important given the potential for cryptic variation to result in disease phenotypes, for example the
56 complex disease phenotypes seen in humans after their recent, rapid change in lifestyle (Felix 2012).
57 The goal of this paper is to provide a proof-of-principle for the presence of abundant cryptic
58 developmental variation in gene expression that is both spatial and quantitative. We focus on the
59 well-known morphogens driving ommatidia specification in *Drosophila* (Li et al. 1995; Raj et al. 2008;
60 Tsachaki and Sprecher 2011; Atkins et al. 2013; Shah et al. 2016).

61 Here we use hybridization chain reaction (HCR) to quantitatively measure spatial gene expression
62 in multiple genotypes from two sexes of two species (*Drosophila melanogaster* and *D. simulans*). We

63 do this in the eye imaginal disc, which is initially patterned by a wave of differentiation marked by a
64 visible indentation of the tissue, termed the morphogenetic furrow (MF), that passes from the posterior
65 to the anterior of the disc, giving each disc an element of both time and space in development (Fig 1).
66 The furrow is initiated by *hedgehog,* which both represses (short range) and activates (long range)
67 *hairy* (Fig 1) (Felsenfeld and Kennison 1995; Strutt and Mlodzik 1997). *hedgehog* also activates the
68 expression of *atonal*, driving the furrow anteriorly (Heberlein et al. 1993; Ma et al. 1993; Struhl 1999).
69 *Delta/Notch* is under the control of *hedgehog* and represses *atonal* (Firth and Baker 2005; Gavish et
70 al. 2016). We analyze the spatial quantitative expression of *hedgehog*, *hairy*, *atonal*, and *Delta* to
71 understand the evolving cryptic regulatory logic of the gene network and cryptic changes in spatial
72 dynamics between sexes and species.
73 HCR is unique in that it produces gene expression patterns that are both quantitative and spatial
74 (Supplementary Materials). The DNA probes were designed and synthesized by Molecular
75 Instruments (Choi et al. 2014) (Table S1). Four genes were multiplexed in each preparation as
76 orthogonally-designed hairpins allowed the simultaneous amplification of their target sequences (Fig
77 1, S1). Eye imaginal discs were dissected using standard procedures from three genotypes and both
78 sexes of previously published *D. melanogaster* and *D. simulans* inbred panels (Mackay et al. 2012;
79 Signor, et al. *in review*). In order to compare gene expression patterns between eye imaginal discs
80 gene expression was quantified and each image was registered to a common grid such that the same
81 regions are being compared despite variation in disc size and deformation (Supplementary Materials).
82 We interpret cryptic variation in quantitative spatial patterns of gene expression in light of
83 regulatory relationships among them. We approach a spatial and quantitative analysis of these gene
84 expression patterns in three ways, first by explicitly creating a spatial gene expression profile and
85 comparing between genotypes, sexes, and species. Second, we were interested in examining if the
86 regulatory relationship between these genes had evolved between species or harbors variation within
87 a species. Lastly, we investigated the possibility that the spatial relationship between these genes
88 relative to the MF had evolved or harbors variation within populations.

First, to characterize the spatiotemporal dynamics of transcriptional activity along the anterior-posterior axis, we took the spatial average of signal across the dorsal-ventral axis and compared between species, genotypes, and sexes (Fig 2). We fit curves to each gene expression profile with mgcv in R, using a generalized additive model with integrated smoothness estimation (Fig 2, Supplemental Materials). Smoothing terms are represented using penalized regression splines. The curves were fit to the original range of values and down sampled to eight points to capture their overall profile.

We found abundant cryptic spatial quantitative variation in expression profiles (Fig 2). The expression profile of *hairy* around the morphogenetic furrow harbors cryptic variation between genotypes and there is an interaction between genotype and sex (Fig 2B & D, $p = 2 \times 10^{-3}$, $p = 0.02$). There is also been cryptic variation between species (Fig 2B & D, $p = 3 \times 10^{-4}$). While *atonal* has not evolved between species, there is cryptic variation in expression profile between genotypes, sexes, and there is an interaction between genotype and sex (Fig 2C & D, $p = 4 \times 10^{-4}$, $p = .02$, $p = .02$). Surprisingly, given the conservation of *Delta* in general, *Delta* harbors variation in spatial quantitative expression behind the furrow between genotypes and sexes (Fig 2D, $p = 2 \times 10^{-3}$, $p = 7 \times 10^{-4}$) and there are significant interactions between genotype and sex (Fig 2D, $p = 2 \times 10^{-3}$). There has also been evolution of *Delta* between species, and evolution of the interaction between species and sex ($p = .03$, $p = 3 \times 10^{-4}$). *hedgehog* is not different between species but is significantly different between genotypes, sexes, and there is an interaction between the two ($p = 5 \times 10^{-4}$, $p = .05$, $p = 1 \times 10^{-3}$). There is also a significant interaction between species and sex ($p = .01$).

Thus, *hairy* and *Delta* have evolved different spatial quantitative expression patterns between species, while *hairy*, *atonal*, *Delta*, and *hedgehog* harbor variation within species and sexes. Given that there are regulatory relationships between these genes, it is interesting to see that they do not all harbor variation for the same factors. This could potentially be due to the influence of other unmeasured regulatory factors, or to variation in the relationship between these genes and other components in the gene regulatory network.

In the previous analysis, we investigated variation in the cryptic spatial quantitative expression pattern of genes in the morphogenetic furrow. Here, we will investigate the possibility that genes in the MF have evolved, or harbor variation, for how they affect each other in particular cells, termed here the 'cryptic variation in regulatory logic'. For example, is high *atonal* expression associated with high expression of *hedgehog*, given that *hedgehog* activates *atonal*? We used the following equations to determine the relationship between the expression of these genes:

$$hairy(i,s) = k^{Dl} \times Dl(i,s) + k^{hh} \times hh(i,s) + \alpha$$

$$atonal(i,s) = k^{Dl} \times Dl(i,s) + k^{hh} \times hh(i,s) + \alpha$$

The coefficient k and constant α were fit using standard methods for multiple regression. Here $hairy(i,s)$ and $atonal(i,s)$ are the measured expression level of each gene in cell i in individual s. $Dl(i,s)$ and $hh(i,s)$ are vectors containing the corresponding expression levels of *hairy* and *atonal*'s regulators *Delta* and *hedgehog*. To determine if the regulatory logic is the same between genotypes and species we can then use the regression coefficients from these models in a MANOVA. We note that because we cannot parse overall changes in spatial expression pattern from cryptic variation in regulatory logic, we focus on genes that are known to be regulated by the other genes in the pathway, i.e. *hairy*, *Delta*, and *hedgehog*. We also cannot exclude the possibility that other unmeasured genes are responsible for producing this variation.

There is cryptic variation in regulatory logic in *hairy* and its upstream regulators *Delta* and *hedgehog* between species and sexes (Fig 3A-C, p = .03, p = .03). We illustrate this difference between species in Figure 3, where a different relationship between *hairy* and *hedgehog* is visible between *D. melanogaster* and *D. simulans*. The regulatory logic of *atonal* does not seem strictly to have evolved between species, but there is a significant interaction between species and sex ($p = 1 \times 10^{-3}$). Furthermore, while there was no significant effect of genotype for *hairy*, there is for *atonal*, indicating that there is cryptic variation segregating in the population effecting the relationship between

142 *atonal, hedgehog*, and *Delta* (p = 1.6 x 10^{-5}). There is also a significant interaction between genotype
143 and sex (p = 1 x 10^{-3}). Thus, the relationship between *hairy* and *atonal* and their regulators has
144 evolved between species and sexes in *hairy*, and between genotypes and sex in *atonal*. For example,
145 in *D. simulans* this appears as an approximately linear relationship between *hairy* and *hedgehog*
146 expression that is not present in *D. melanogaster* (Fig 3A).

147 Cryptic variation in regulatory logic between sexes and species is somewhat surprising. Eye size
148 does seem to vary both within and between species, but the exact nature of this variation is unclear.
149 By one account female eyes in *D. melanogaster* are smaller than expected based on allometry, while
150 other work finds that the opposite is the case in both *D. melanogaster* and *D. simulans* (Cowley and
151 Atchley 1988; Posnien et al. 2012). Body size is at least in part controlled by *Sex-lethal,* and sex
152 specific development in somatic tissues are governed by *doublesex* (Rideout et al. 2010; Mathews et
153 al. 2017). We have detected *doublesex* expression in the eye anterior to the morphogenetic furrow, in
154 cells prior to cell cycle arrest at the furrow (Fig S2). It is possible that an interaction between
155 *doublesex* and *hairy*, *atonal*, *Delta*, or *hedgehog* is mediating the change in cryptic variation in
156 regulatory logic between sexes. It is also possible that some of this variation is not cryptic, and in fact
157 has an effect on sex-specific development of the eyes. To date very little sexual dimorphism has been
158 documented in *Drosophila* eyes, other than a > 10% difference in the composition of ommatidial
159 subtypes in some genotypes (Hilbrant et al. 2014).

160 Lastly, we wanted to understand if there is cryptic variation for the relationship between the furrow
161 and gene expression, or if variation existed for the size of the furrow overall. For example, the furrow
162 was called as the position of overlap between *atonal* and *hairy* expression, but it is unclear how the
163 overall gene expression pattern of these genes relates to their overlap. For example, is the position of
164 maximum expression of each always the same relative to the furrow? Two processes occurred to
165 make the furrow comparable between samples, they were shifted to occupy the same position
166 depending upon the position of overlap of *hairy* and *atonal*, and they were scaled to occupy the same
167 total area. The amount required to scale will depend both on the size of the original disc and the width
168 of the furrow relative to the disc. To account for differences in size we include the number of rows in

169 the original disc prior to any transformations as a cofactor and perform ANOVA in R. The amount that
170 they were shifted is not significant for genotype, sex, or species, suggesting that the relationship of
171 maximum gene expression with the furrow does not vary. However, the amount that they were scaled
172 is, after accounting for original differences in size, between species ($p = 1.38 \times 10^{-6}$). This suggests
173 that the total relative width of the MF varies between species, but not between genotypes or sexes.
174 This is also suggestive of evolving interrelationships among genes that could result in broader or
175 narrower areas in which they enhance or suppress expression of one another.
176 In the past, the developmental approach to understanding gene networks has been to analyze
177 large effect mutations and their qualitative downstream effects. This results in robust data on
178 perturbed regulatory networks, but obfuscates information on more normal interrelationships among
179 genes. Similarly, the evolutionary approach to development generally targets large changes that have
180 occurred over broad phylogenetic distances (Signor et al. 2016). Here we take an entirely different
181 approach by focusing on small, quantitative variation between genotypes and closely related species.
182 Using this approach, we were able to quantify the regulatory relationships among genes and to
183 observe how these relationships are altered between sexes and how they evolve between sister taxa.
184 Reconciling the speed of this cryptic developmental evolution with the patterns of molecular evolution
185 in the underlying genes will yield new insights on the rules governing microevolution of gene
186 regulatory networks.
187
188 **AUTHOR CONTRIBUTIONS**: S. A. performed the staining and imaging of the eye discs, K. K.
189 processed the imaging data, S.S. conceived of the experiment, analyzed the processed data and
190 wrote the paper, S. N. conceived of the experiment, coordinated the research, and co-wrote the paper.
191
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195

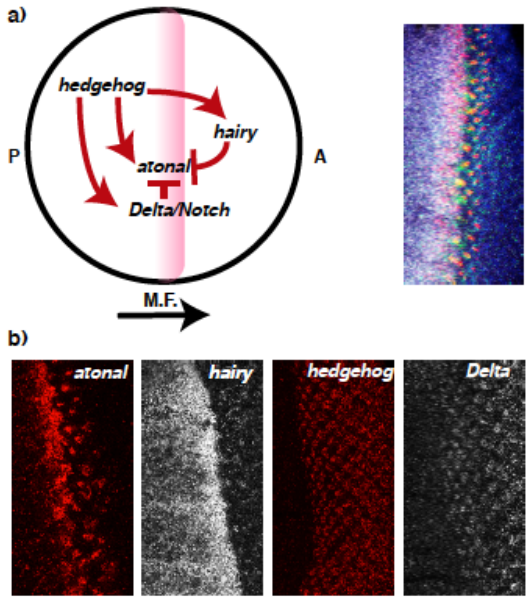
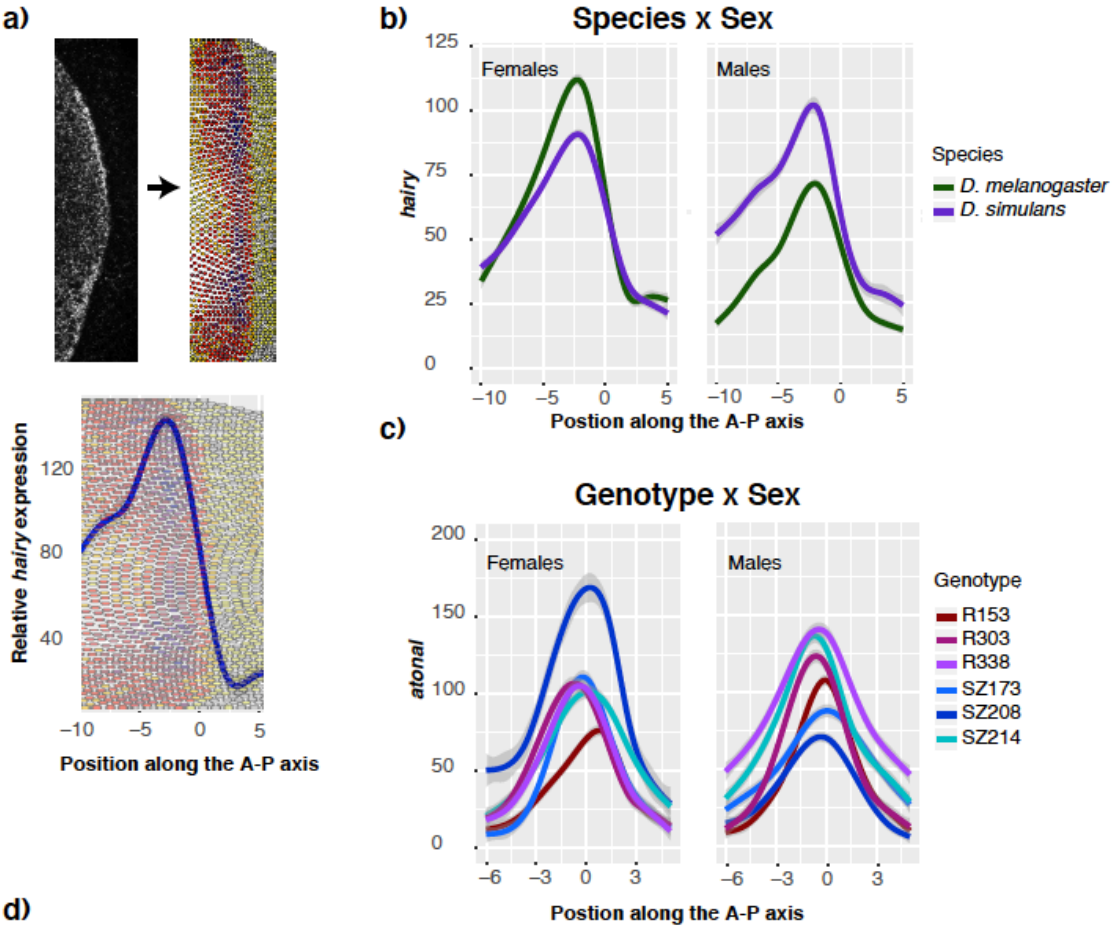


Figure 1. **a)** A summary of the eye patterning genes and pathway explored in this paper. The position of the morphogenetic furrow is shown in pink, and its direction of movement indicated below. Regulatory relationships are illustrated either as repression (bar) or activation (arrow). Shown at right is a composite image of all four genes (*hairy* (white), *atonal* (red), *Delta* (green), and *hedgehog* (blue)). Please note that this is a very simplified illustration, intended as a summary between the genes that are the subject of this paper rather than an exhaustive illustration of the eye patterning gene network. **b)** Example gene expression patterns of each gene. *hedgehog* and *Delta* were recolored in Photoshop (Adobe, v. 21.1) without altering any aspects of expression level or contrast.



hairy

Effect	*numDF*	*denDF*	F-value	*p*-value
Species	8	35	5.1	**3×10^{-4}**
Genotype	32	152	2.07	**2×10^{-3}**
Sex	8	35	1.08	0.32
Species x Sex	8	35	1.94	0.08
Genotype x Sex	32	152	1.73	**0.02**

atonal

Effect	*numDF*	*denDF*	F-value	*p*-value
Species	8	35	1.53	0.18
Genotype	32	152	2.31	**4×10^{-4}**
Sex	8	35	2.6	**0.02**
Species x Sex	8	35	1.91	0.09
Genotype x Sex	32	152	1.73	**0.02**

Delta

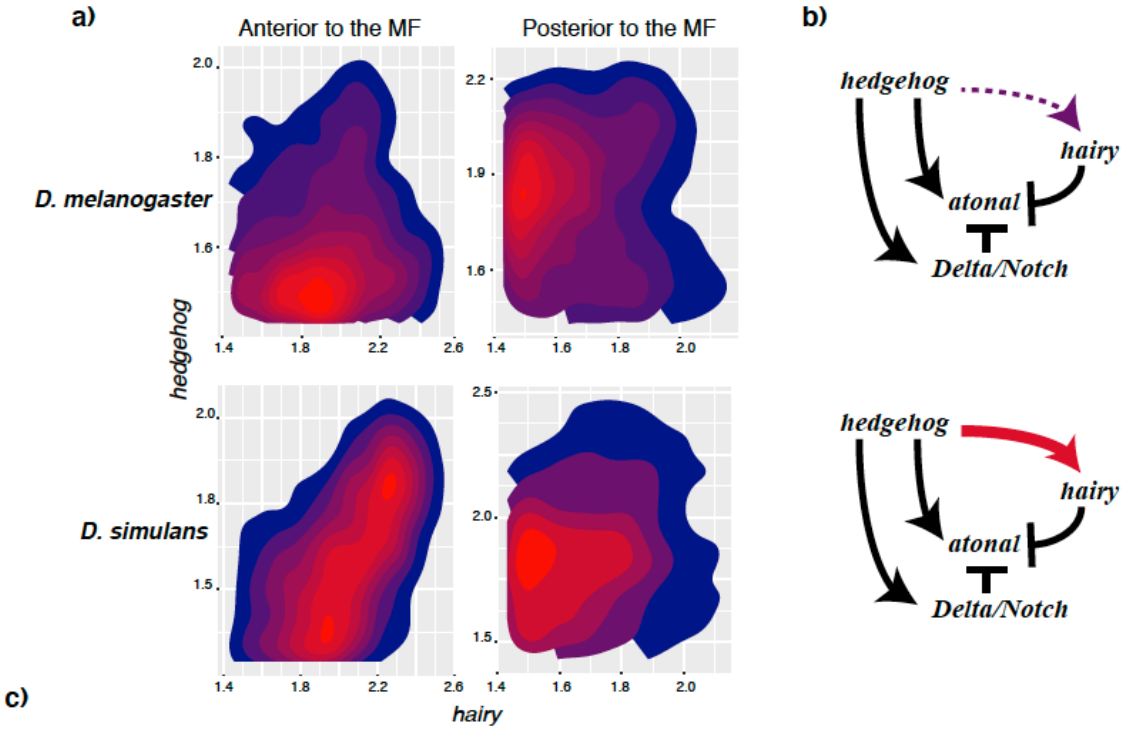
Effect	*numDF*	*denDF*	F-value	*p*-value
Species	8	35	2.43	**0.03**
Genotype	32	152	2.07	**2×10^{-3}**
Sex	8	35	4.55	**7×10^{-4}**
Species x Sex	8	35	5.03	**3×10^{-4}**
Genotype x Sex	32	152	2.03	**2×10^{-3}**

hedgehog

Effect	*numDF*	*denDF*	F-value	*p*-value
Species	8	35	0.87	0.55
Genotype	32	152	2.28	**5×10^{-4}**
Sex	8	35	2.23	**0.05**
Species x Sex	8	35	2.88	**0.01**
Genotype x Sex	32	152	2.17	**1×10^{-3}**

Figure 2. **a)** An example of the data-processing used to produce gene expression profiles. *hairy* expression is shown on the left for an imaginal disc, while on the right is the same disc after it has

been segmented and registered. The bottom is an example of a curve being fitted to the gene expression profiles, though note that the curve corresponds to the average in a given row (*x*-axis). **b)** An illustration of variation in hairy expression between species and sexes. Curves shown are fitted to all genotypes within a sex and species with confidence intervals indicated in gray. Only the effect of species was large enough to be significant, though it is clear that perhaps with a larger sample size an interaction effect would also have been significant. **c)** An illustration of variation in *atonal* expression between genotypes and sexes. Curves shown are fitted to all samples within a genotype and sex with confidence intervals indicated in gray. The effect of genotype, sex, and genotype x sex were significant. All *D. melanogaster* genotypes are shown in shades of purple, *D. simulans* in blue. **d)** The results of the full model for each gene, significant *p*-values are indicated and bold, with gray shading.



c)

hairy					*atonal*				
Effect	***numDF***	***denDF***	**F-value**	***p*-value**	**Effect**	***numDF***	***denDF***	**F-value**	***p*-value**
Species	2	41	4.03	**0.03**	Species	2	41	2.64	0.08
Genotype	8	84	1.2	0.31	Genotype	8	84	5.43	**1.6×10^{-5}**
Sex	2	41	3.95	**0.03**	Sex	2	41	0.67	0.52
Species x Sex	2	41	0.78	0.46	Species x Sex	2	41	8.09	**1×10^{-3}**
Genotype x Sex	8	84	1.11	0.37	Genotype x Sex	8	84	2.41	**0.02**

Figure 3. **a)** An example of cryptic variation in regulatory logic between *D. simulans* and *D.*

220 *melanogaster* for *hairy* and *hedgehog*. The heat map illustrates the density of points, and thus reflects
221 the frequency of a given co-expression profile between *hairy* and *hedgehog*. Gene expression values
222 were log-transformed to better illustrate lower values and split between anterior to the MF and
223 posterior to the MF. The split between the two regions was to investigate the possibility that *hedgehog*
224 had a different regulatory relationship with *hairy* depending upon its relationship to the furrow, given
225 that *hedgehog* is thought to activate *hairy* long range and repress *hairy* short range. Indeed, anterior
226 to the MF *D. simulans* has an almost linear relationship between *hairy* and *hedgehog*, while in *D.*
227 *melanogaster* almost any value of *hedgehog* can be associated with high *hairy* expression. **b)** An
228 illustration of the change in the relationship between the expression of *hedgehog* and *hairy* between
229 *D. simulans* and *D. melanogaster* anterior to the MF. The dotted, darker line is meant to indicate less
230 of a regulatory relationship than the thick red line. c) The results of the full model investigating
231 changes in the regulatory relationship between *hairy*, *Delta*, and *hedgehog*, and *atonal*, *Delta*, and
232 *hedgehog*.

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