**Abstract**

Formation of patterns that are proportional to embryo size is an intriguing and poorly-understood feature of development. Molecular mechanisms for achieving such proportionality, or scaling, can be probed by evaluating the quantitative properties of morphogen gradients. Recent investigations of the Drosophila morphogen gradient Bicoid (Bcd), which instructs pattern formation along the anterior-posterior (AP) axis, have uncovered two distinct mechanisms of scaling. While between-species scaling is achieved through scaling the length constant () of the Bcd gradient profile with embryo length, within-species scaling is through scaling its concentration at the anterior (B0) with embryo volume. Both mechanisms allow the Bcd gradient profile in large embryos to “reach” a longer absolute distance from the anterior. Here we investigate whether the between-species Bcd scaling mechanism may also be utilized by embryos within a species in specific cases. We quantify Bcd gradient properties in embryos from a pair of Drosophila melanogaster lines that were force-selected to have large and small eggs. Our results reveal that the Bcd gradient profiles in these embryos have  values that are scaled with embryo length. While the amount of bcd mRNA in the large embryos is more than in the small embryos as expected, its distribution is uncharacteristically diffused in the anterior. We suggest that the altered bcd mRNA distribution in the large embryos is responsible for our observed reduction in B0 and increase in  Need a conclusion.

**Introduction** (rationale or framework of how we present the current work)

>>Dev paper: within-species scaling is achieved by a mechanism of volume dependent deposition of bcd mRNA

>>the volume dependent deposition of bcd mRNA is a general effect, confirmed by multiple lines/pairs tested

>>lambda in absolute length is the same (in large and small embryos) for within-species scaling—but higher B0 in large embryos allows the gradient to reach further (in absolute length) to achieve scaling

>>Gregor paper: between-species scaling is achieved by having lambda scaled with L…this is a mechanism distinct from our documented within-species scaling mechanism

>>Question: can within-species scaling also be achieved through this alternative (distinct) mechanism and if so, how?

>>the rest of the story is simple and set to go—we will provide answer to this question—and that is our paper.

The process of development is fundamentally robust; the achievement of appropriate scaling is evolutionarily critical. An appropriately scaled system must necessarily be insensitive to fluctuations in size. The *Drosophila* syncitial embryo provides a well studied system which describes how pattern formation occurs in an environment potentially variable in size.

The Bicoid morphogenic protein forms an exponentially degrading concentration gradient along the anterior-posterior axis of the early embryo. Previous studies have shown that scaled pattern formation can be achieved in the face of small and large difference in size. The Bicoid protein is an anterior determinant which induces the expression of downstream genes; ultimately directing the formation of the head and thorax structures.

In oour previous work, we demonstrate how scaling could be achieved in both large and small environments through the total aggregate amount of Bcd mRNA desposited maternally during ooogenesis. In the present study, we reveal that the total amount of Bcd mRNA is not the sole determinant by which scaling can be achieved. Here we show that the manner in which Bcd mRNA is distributed is a contributing factor in scale determination, sufficient to support scaled patterning. We find that defects in Bcd mRNA localization genes allow for viability to be permissible in this environment.

**Results** (subtitles)

**1. Bcd staining data in alternate large embryos reveal properties uncharacteristic of the measured size**

>>Show raw images of staining of alternate large/small

>>Analyze data showing/establishing that B0 values are going “the other way”, p value etc. L values etc. (maybe have a table showing all the properties, including the published pair)

>>also show raw images of published large/small side by side. Analyze B0 values—showing essentially that the alternate large is a “bad”, not the alternate small

In the present study we utilized a pair of inbred *Drosophila* lines which have been forcibly selected for egg size in parallel with those we have previously published (Miles ref). Embryos from Line 2.49.3 demonstrate morphogenic behavior which defies the predictions which their size would suggest. Embryos from this line immediately show a marked difference in the appearance of Bicoid when compared to previous studies using similarly selected large embryos. The Bcd concentration at the anterior-most position of the gradient (B0) is \_\_\_\_ (a.u.) in embryos from Line 2.49.3, compared to \_\_\_\_ (a.u.) in embryos from Line 9.31.2. Several previous studies have shown a positive correlation between B0 and Egg Length (EL). However in this pair of embryos, which possess similar physical characteristics, the correlation appears quite distinctly negative. We investigate further in how properties of Line 2.49.3 embryos may differ in other ways.

**2. Alternate large embryos develop scaled patterning**

>>Show hb data, show eve data (comparing between alternate large and alternate small), x, x/L data showing scaling/convergence

In order to directly probe the effect of the altered Bcd profile in Line 2.49.3, we performed a fluorescent in situ hybridization against Hunchback (Hb) mRNA in early embryos. When plotted on the x (µm) axis, the boundary positions for the mean profiles from the two lines are dramatically disparate. However the, two profiles converge when plotted along the x/L axis, for scaled embryo length. This is indicative of proportionality being maintained between these two lines despite the large difference in size.

**3. Bcd gradient profiles in alternate large/small embryos are scaled (through a distinct way)**

>>now back to Bcd--show raw profiles (or alternate large and alternate small) in x, x/L

>>showmean B (for published large and small, alternate large and alternate small in same figure) in x, x/L (see your Dev paper)—convergence etc

>>show delta B, delta x etc (same as your Dev paper)

We performed a immunofluorescence staining against Bicoid protein in embryos from Lines 2.49.3 and 9.31.2. at cycle 14. We found that in the raw Bicoid profiles, the larger embryos from Line 2.49.3 do not conform to our expectations set by our previous work. In prior studies, physically large embryos would suggest that there would be a substantially higher amount of Bcd protein, because it would have to diffuse over a larger area in order to reach appreciable concentration thresholds and activate downstream transcription.

We analyzed the profile data further; we found that the ΔB difference between the two lines to be propagated throughout the embryo when plotted on the absolute length axis. However, this difference becomes abolished when the data is plotted over relative egg length (x/L).

We also interpolated these profile data and calculated the x-position of each profile at various Bcd threshold concentrations.

**4. Scaling of lambda with L in alternate large and small embryos**

>>Show lambda profiles, in x, x/L (we can include the data for published large and small as well, if necessary)

There are two main modes of scaling mechanisms which we have identified; those of which govern the interaction between species and those which mediate scaling within a species. It has been shown previously that scaling in these two conditions are distinct and can be observed through a difference in the length constant lambda, (λ).

**5. A distinct scaling mechanism yields a distinct scaling coefficient S profile**

>>Show S profile (what comparisons? Just alternate pair or include the published pair etc for comparison)

In light of the S profiles looking bad, consider removing this section.

**6. bcd mRNA amount in alternate large remains scaled with egg volume**

>>show raw data and processed data, also include aggregate B data (with our without figure—to be decided)

We decided to continue the investigation further, there must necessarily be a cause for the remarkable anti-correlation of B0 to EL and yet scaling is maintained. In order to look deeper into the issue, we performed an fluorescent in-situ hybridization in early embryos from Line 2.49.3 and 9.31.2, from Cycles 1 to 5, with a dioxygenin-labeled probe against Bicoid mRNA. Surprisingly, using previously established methods for quantification, we found that embryos from Line 2.49.3 actually contain a higher amount of Bcd mRNA than the physically smaller Line 9.31.2.

From Line 2.49.3, paradoxically, the Bcd mRNA amount is consistent with the prediction set by the size of the embryo; however, the result is seemingly diametrically opposed to the amount of Bcd protein found in the gradient profile data. **7. bcd mRNA distribution in alternate large is more diffused**

>>compare alternate large and published large, may also compare with alternate small etc…all for making a point in the subheading

Upon closer inspection, we found that while the total amount of Bcd mRNA is appropriately proportional, the distribution of the mRNA at the anterior tip is significantly larger. To characterize this distribution, we asked whether this was a result of a dose dependency. We performed a FISH, again using digoxygenin labeled Bicoid mRNA probe on embryos from females with 1x, 2x or 3x copies of Bicoid. These data show that the relation between Bcd dosage and area across which it distributed is not strictly dose dependent. The signal size for 1x is significantly smaller, however, we believe that the data from 2x and 3x embryos is more accurately representative of the conditions in Lines 2.49.3 and 9.31.2.

--To keep things simple, do not get into the within line issue.

--we use “alternate large” for now, but we need to find a better term for it

--People will ask about what has been mutated—need to sequence

--People will ask about whether the alternate large are defective in other ways (ts, cuticle etc?)

**Discussion:**

Interestingly, indications of similar trends can be found upon revisiting previously published data. In 2008, He et al. show that the Bcd gradient profile in Staufen embryos, a mutation known to disrupt Bcd mRNA localization, also have a depressed B0 at the anterior tip. Similar to features that we observe in the present study, the length constant lambda in staufen embryos is slightly higher compared to wildtype [105 vs 100]. While Bcd mRNA FISH was not performed on those embryos at the time, we make the assumption that the total amount of Bcd mRNA has not been significantly affected. We predict that the gradient characteristics are determined, in this case, no by the total amount of Bcd mRNA, but rather solely by the distribution. -------------------------------------------------------------------------------------------------------A well-appreciated feature of animal development is the formation of body parts that are proportional to an individual’s overall body size. This is reflective of the robustness of the developmental process. A full understanding of the molecular underpinnings of scaling requires insights into development at two distinct levels: specification of scaled patterns by morphogen gradients and coordinated growth of organs/tissues. Our current work focuses on the former. Here, we evaluate the properties of the morphogen gradient of Bcd in embryos from Drosophila lines that had been selected specifically to have large and small eggs. Since Drosophila embryo itself does not grow in size during the action time of the Bcd morphogen, our model system thus makes it possible to investigate the scaling problem exclusively at the level of pattern specification without an entanglement with tissue/organ growth. Our results described here and in another report (ref) document the value of combining quantitative methods and natural genetic variants in uncovering mechanistic insights into morphogen gradientproperties in specifying scaled patterns.

In our current study, we took advantage of a pair of selected Drosophila lines where the large and small embryos that exhibit Bcd gradient properties contrasting with those reported earlier (ref). Our results suggest an alternative mechanism of within-species scaling for the Bcd gradient. This mechanism complements the established within-species mechanism and, at a gross level, bears resemblance to the reported between-species scaling mechanism (ref). Specifically, our results show that, although the large embryos have more maternally deposited bcd mRNA than the small embryos as expected, the mRNA distribution is more diffused in the anterior. It is well documented that bcd mRNA that is not strictly localized to the anterior is subject to translational inhibition by nanos (ref). Thus the diffused bcd mRNA distributions in the large embryos can readily explain our observed Bcd gradient properties with regard to both a lower-than expected B0 and a larger-than-expected lambda (in absolute length). An alternative model to explain these unexpected properties of the Bcd gradient profile in the large embryos, which we currently do not favor, would envoke an altered diffusivity and degradation of Bcd in these embryos. Regardless of precisely how the Bcd gradient profile in the large embryos is achieved, it is important to emphasize that a critical finding here is the documentation of the existence of such a profile to exemplify a distinct within-species scaling mechanism for Bcd. Genetic investigations of this large line may yield useful insights relevant to ourdeeper understanding of the molecular mechanisms of scaling at the level of Bcd gradient formation.

The two within-species mechanisms reported here and previously (ref) are different in several aspects. While both mechanisms allow Bcd gradient profiles in large and small embryos to converge near mid embryo, the range of convergence is much greater for the currently reported mechanism (correct? What are the differences and similarities that can be quantified??). It has been proposed recently that, based on our finding that hb transcription is quickly shut down at early cycle 14, Bcd may be able to directly pass its scaling properties to hb expression near mid embryo. Thus, while hb scaling may be achieved similarly under the two different scenarios, scaling in other parts of the embryos likely depends on the operation of additional mechanisms. In this context, it is important to note a difference between how the large embryos studied here and previously achieve Bcd gradient scaling: their anterior parts exhibit opposite properties (higher or lower Bcd concentrations) relative to their small counterparts. These results suggest that additionalmechanisms responsible for scaling along the AP axis may have been adjusted in the large embryos studied in this report. We emphasize that, despite these differences, egg size-dependent maternal deposition of bcd mRNA is conserved in all the embryos we have studied. This suggests that the artificial selection process for all the lines that we have analyzed did not alter the oogenesisprocess with regard to the egg volume-dependent maternal deposition of bcd mRNA to the egg.

Fig 1. <Figure Title – Bcd raw data> (A-D) Representative embryos from Lines 2.49.3 and 9.31.2 which have been stained for Bicoid (A,C) and counterstained by DAPI (B,D). Images are from the midsagittal section. (E-H) Fluorescence intensity data extracted to show the Bcd protein gradient profile plotted over absolute length (E, G) and relative egg length (F, H). Also shown are mean profiles from the *bcdE1* null mutant to be used for background subtraction. (n = x and y, respectively)

Fig 2. Mean Bicoid protein gradient profiles of Lines 2.49.3 and 9.31.2 demonstrate convergence. (A, B) The mean profiles were plotted over absolute length (A) and relative egg length (B).

Fig 3. Within-line scaling is lost for embryos from Line 2.49.3. (A, B) Profile data from these embryos were bifurcated according to egg length plotted over absolute length (A) and relative egg length (B).

Fig 4. Aggregate Bcd fluorescence from mean profiles. (A) Shown here are the sum of Bcd fluorescence intensities from the mean profiles from Line 2.49.3 (Blue) and Line 9.31.2 (Red). (A, inset) Recorded values of the aggregate intensities.

Fig 5. <Figure Title 2 – Hb raw data> (A-D) Shown here are raw Hunchback profiles from fluorescent in situ hybridization in embryos from Line 2.49.3 and 9.31.2 plotted over absolute length (A, C) and relative egg length (B, D).

Fig 6. Convergence of mean Hunchback profiles are indicative of scaling. (A, B) Shown here are mean profiles from Hunchback in situ data plotted over absolute length (A) and relative egg length (B).

Fig 7. Bicoid mRNA fluorescent in situ hybridization in Lines 2.49.3 and 9.31.2 reveal disparities in distribution of maternally deposited products. (A) Shown here are raw immunofluorescent images of Bicoid mRNA in early embryos; contours outlining the embryo (Green), specific signal (Blue) and posterior reciprocal (Red) are overlaid. (see text)

Fig 8. Bicoid mRNA intensity is correlated with embryo size. (A) Shown here are aggregated fluorescence intensity values of Bcd mRNA in early embryos plotted over egg length (A) and estimated egg volume (B). [Also shown here are previously published intensity values from similar lines (tbd, will have to convert to relative dose or convert from intensity to relative dose to intensity via 2 std curves)].

Fig 9. Bicoid mRNA contour area is significantly larger in Line 2.49.3. (A-C) Shown here are the total areas from the Bicoid in situ hybridization specific signal bounded by calculated contour lines plotted over egg length (A), estimated egg volume (B), and Egg Area[dimensions can be directly compared; functionally meaningless] (C).

Fig S1. <Figure Title 3 – Genomic Sequencing Data> TBD

Fig S2. <Figure Title 4 - Cuticle> TBD

Fig S3 <Figure Title 5 – Even-skipped> Eve data – C. Miles. [Can be moved to main section or cut entirely, tbd].