**Adjusting the shape characteristics of the Bicoid gradient profile in**

**Drosophila melanogaster embryos for size scaling**

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**Abstract**

Formation of patterns that are proportional to embryo size is an intriguing but poorly-understood feature of development. Molecular mechanisms controlling such proportionality, or scaling, can be probed through quantitative interrogations of the properties of morphogen gradients that instruct patterning. Recent studies of the Drosophila morphogen gradient Bicoid (Bcd), which is required for anterior-posterior (AP) patterning in early embryos, have uncovered two distinct mechanisms of scaling. Between-species scaling is achieved by adjusting the exponential characteristics of the Bcd gradient profile, namely, its length constant (). In contrast, within-species scaling is achieved through adjusting the amplitude of the gradient profile, namely, the Bcd concentration at the anterior (B0). Both mechanisms allow the Bcd gradient profile in large embryos to “reach” a longer absolute distance from the anterior. Here we investigate whether the mechanism of adjusting the shape characteristics of the Bcd gradient profile may also be utilized for within-species scaling. We quantify Bcd gradient properties in embryos from a pair of Drosophila melanogaster lines that were artificially selected to have large and small eggs. We find the large embryos have an unexpectedly lower B0 than the small embryos, but have a length constant  that is scaled with embryo length but is uncharacteristically large for Drosophila melanogaster. We show that, while the large embryos have more bcd mRNA than the small embryos as expected, bcd mRNA distribution in these large embryos is diffused. Our results suggest that, during the artificial selection and inbreeding process, the large-egg line has acquired properties uncharacteristic of normal Drosophila melanogaster in order to maintain a scaled Bcd gradient in the embryo. Our study thus documents, for the first time, a case of within-species Bcd scaling achieved through adjusting the profile’s exponential characteristics.

**Introduction**

A striking feature of animal development is the formation of body parts that are proportional to an individual’s overall body size (ref). This is reflective of the robust nature of the developmental process, which can “correct” inevitable variations at all levels relevant to development, ranging from molecular to organismal and environmental levels. A full understanding of the molecular underpinnings of scaling requires a mechanistic knowledge about two distinct aspects of development: specification of scaled patterns and coordinated growth of organs/tissues of an individual. Recent quantitative studies have uncovered insights into scaled patterning specification (ref), which is the focus of this work. A well-documented feature of patterning is the involvement of regulatory networks. By nature, complex networks can confer robustness to a patterning system, stemming from the regulatory power of, e.g., feedback loops and cross-regulatory circuits (ref). Recent studies of the Drosophila morphogen gradient Bicoid (Bcd) show that the initial (maternal) input to a patterning system already exhibits scaling properties (Greg, Bergman, Cheung, He). These results suggest that investigating morphogen gradient properties can benefit our understanding developmental robustness.

The morphogen gradient of Bcd is required for patterning along the anterior-posterior (AP) axis in early Drosophila embryos (ref). The protein gradient is derived from the anteriorly localized, maternally-deposited bcd mRNA (ref). While details of Bcd gradient formation remain an active topic of investigation (ref), it is generally thought that this process can be described by a diffusion model, where Bcd protein synthesized at the anterior diffuses and decays throughout the embryo (ref). In this simple diffusion model, the steady state profile of the Bcd concentration B follows an exponential function of distance x from the anterior: B = A e-x/, where A is the amplitude and  is the length constant. Since bcd mRNA, the source for Bcd protein, is not localized to a single point in the actual embryo as in the idealized diffusion model (Gregor, Spirov, Cheung), the anterior part of the experimentally measured Bcd profile deviates from this exponential shape (Deng, Bergman model). For an experimentally measured Bcd profile, its concentration at the anterior, B0, can be used as a substitute for amplitude (ref). In Drosophila melanogaster, the length constant  of the observed Bcd concentration gradient is in the range of ~100 m (ref). This characteristic length constant is constrained by the effective diffusivity and stability of Bcd in the early embryo of this species (Gregor PNAS, Liu-two refs, Wieschaus Bioph J, Dostni ref).

Studies of Bcd gradient properties have uncovered two distinct ways to scale the Bcd gradient profile with embryo size (ref). In both cases, the Bcd gradient profiles from large and small embryos become “similar” to each other as a function of relative AP position or fractional embryo length x/L. Thus, at a given relative AP position near the middle section of the embryo, Bcd gradient scaling allows large and small embryos to have comparable Bcd concentrations regardless of their size. The two distinct ways for Bcd gradient scaling were proposed to operate for embryos from different species or for embryos within a species (ref). While between-species scaling is achieved by scaling with L (ref), within-species scaling is achieved by scaling B0 with egg volume (ref). Here, we report our quantitative studies of the Bcd gradient properties in embryos from a pair of Drosophila inbred lines that had been artificially selected for egg size (ref). We show that, while the large embryos have a B0 that is uncharacteristic low for the embryo size, its length constant is uncharacteristically large for Drosophila melanogaster. We show that this large  is scaled with the large L in these embryos. These results document for the first time a within-species scaling for Bcd through adjusting the profile’s exponential shape characteristics. We further show that the large embryos have a more diffused distribution of bcd mRNA. These results suggest that, under an extended artificial selection and inbreeding process, properties that are uncharacteristic of normal Drosophila melanogaster can be acquired in order for the embryo to maintain a scaled Bcd gradient profile.

**Results**

**Embryos from a pair of selected Drosophila melanogaster lines exhibit Bcd properties uncharacteristic of their size**

In a recent study (ref), we quantified the Bcd gradient and bcd mRNA properties in embryos from a pair of inbred *Drosophila* lines which had been artificially selected for egg size (Miles ref). We also analyzed embryos from several pairs of artificially selected populations that had not gone through the inbreeding process. From those experiments, a general “rule” emerged that large embryos contain more maternally deposited bcd mRNA, which led to a higher concentration of Bcd at the anterior B0 and scaling of the Bcd gradient profile (ref). In analyzing another pair of inbred lines (referred to as the “alternative pair” to simply make a distinction with the original inbred pair that we reported previously—see Methods for details), we found that the embryos exhibited Bcd properties uncharacteristic of their size, as further detailed below. Here we used quantitative immunofluorescence staining to detect Bcd in whole mount embryos (see Methods for details). Our staining and imaging procedures maintained a linear relationship between the captured fluorescence intensity and Bcd concentration (see Methods). We thus use fluorescence intensity and Bcd concentration (both in arbitrary units) interchangeably in this report.

Fig. X shows raw fluorescence images of the large and small embryos (all large and small embryos in this report refer to those from the alternate pair of inbred lines unless otherwise specified). The image of the large embryo has visibly lower fluorescence intensity in the anterior than the small embryo. Quantification of the intensity data revealed that B0 is ?? in large embryos (n =??), which is much lower than that in small embryos (n =??), ?? (p = ??). These Bcd concentration properties contrast with the measured lengths of these embryos: ?? and ?? for large and small embryos, respectively (p =??), demonstrating that these embryos have Bcd gradient properties uncharacteristic of their size.

Despite the unexpectedly “reversed” B0 properties in the large and small embryos, the hb expression boundary positions are scaled with embryo lengths. Here, we performed quantitative fluorescence in situ hybridization (FISH) detecting mature hb mRNA in whole mount embryos (see Methods for details). We found that the hb boundary position, xhb, which is defined as the AP position at which hb mRNA level is at half maximal, is ?? and ?? for large and small embryos, respectively (p = ??; see Fig. X for hb FISH intensity profiles as a function of absolute distance from the anterior x). When the hb boundary positions in these embryos are measured as normalized AP position or fractional embryo length x/L, these embryos have (largely?? Or really??) similar hb boundary positions (?? And ?? for large and small embryos, respectively, p =??; see Fig. X for hb profiles as a function of x/L). These results suggest that investigating the Bcd gradient properties in these embryos may help reveal useful insights relevant to our understanding of scaled patterning.

**Bcd gradient profiles in the large and small embryos exhibit scaling properties**

To quantitatively evaluate the Bcd gradient profiles from the large and small embryos, we extracted the Bcd intensity B from individual embryos and expressed it as a function of either x or x/L (see Methods). Fig. X shows the mean B profiles from the large and small embryos as a function of x. These results further confirmed the observation that the anterior region of the large embryos has lower Bcd concentration than their small counterparts. When these mean B profiles are expressed as a function of x/L (Fig. X), they exhibited a striking convergence in most parts of the embryos along the AP axis (except the anterior). The observed convergence is a hallmark of Bcd gradient scaling (ref).

To better evaluate this convergence or scaling of Bcd gradient profiles, we plotted the difference between the mean B values of these embryos, B, as a function of either x or x/L (Fig. X and Y respectively). Our results show that, despite the low mean B in large embryos at the anterior region yielding a negative B there, the two mean profiles cross each other at x = ~100 m (Fig X) to yield a positive B. This positive B is propagated relatively stably through the rest of AP length (Fig. X). Fig. X shows that B, when plotted as a function of x/L, was greatly reduced to hug around the 0 line in most parts of the embryo (except the anterior). These results demonstrate quantitatively that the mean Bcd gradient profiles from the large and small embryos are scaled in most parts of the embryos (except the anterior region). They show that these two profiles are “similar” to each other when expressed as a function of normalized AP position.

To further evaluate the scaling properties of the Bcd gradient profiles in the large and small embryos, we plotted the difference between large and small embryos in positions at which the mean Bcd profiles cross given thresholds. This difference is defined as x and is plotted either as a function of x or x/L. (see Dev paper for how to continue the rest)>/. Together, our results document that, despite the unexpectedly “reversed” B0 in the large and small embryos, the Bcd gradient profiles are actually scaled in most parts of the embryo along the AP axis.

**Bcd gradient scaling in the large embryos through scaling the length constant with embryo length**

The unexpectedly “reversed” B0 but scaled Bcd gradient profiles in the large and small embryos suggest that either the large embryos or the small embryos (or both) have properties that are uncharacteristic of their size. We suspected, based on our experience of “looking at” the Bcd gradient profiles, that the large embryos were somewhat “abnormal”. We noted that, despite the low B0 in the large embryos, the mean Bcd gradient profile expressed as a function of x actually crosses over with that from the small embryos at ~100 m. These results suggest that the shape characteristics of the Bcd gradient profiles are different between the large and small embryos. For an exponential gradient such as that of Bcd, the crucial characteristic of its shape is length constant , which quantifies how “quickly” the Bcd concentration drops off as a function of distance from the anterior. To evaluate the Bcd gradient profiles in large and small embryos, we calculated from individual embryos. We reasoned that, since the Bcd gradient profile in Drosophila melanogaster has a well documented consensus value of ~100 m (ref), obtaining the values in the large and small embryos may give us further insights into which of these profiles (or both) is “abnormal”. Our results show that the large and small embryos have an average  of ?? and ?? m, respectively (p = ??). These results show that, while  in the small embryos appears “normal”,  in the large embryos is uncharacteristically large for Drosophila melanogaster embryos.

The uncharacteristically large  in the large embryos is scaled with embryo length. Specifically, when length constant was calculated as a relative distance /L, we found that the large and small embryos have (largely? Or really?) similar values (?? And ?? for large and small embryos, p = ??). To further evaluate the Bcd gradient profiles and provide a visual presentation of the differences in their shape characteristics between large and small embryos, we plot the mean B profiles on ln scale. Specifically we plot lnB/Bmax as a function of x or x/L. Ln conversion makes ??? slope is ??. see Dev paper and fill in the blank. Figs. X shows that the two linear fits of the lnB/Bmax plots have distinct slopes, with the large embryos having much less steep. This further illustrates that the large embryos have a large  value in absolute distance. However, when the lnB/Bmax profiles were plotted against x/L, these slopes become similar, further demonstrating that that these embryos have relative length constant values that are similar to each other. These results show that the large embryos achieve Bcd gradient scaling by scaling the length constant with embryo length, a documented strategy employed only for between-species scaling (ref).

**The large embryos have more bcd mRNA than the small embryos**

The uncharacteristically low B0 in the large embryos raised an important question with regard to the amount of maternally deposited bcd mRNA. To gain insights into this question, we performed a FISH in whole mount early embryos to detect bcd mRNA (see Methods). Describe more details about technique, linearity, calibrating, Otsu etc. What controls etc? Values, p etc. Egg length, volume etc. compared between large and small? Still follow proportionality (between amount and volume?) The rest of this section should be simple now.

**Diffused bcd mRNA distribution in the large embryos**

Both the low B0 and large in the large embryos suggest “abnormal” properties relevant to Bcd gradient formation. According to a simple diffusion model,  of a steady state exponential gradient is a function of the diffusion constant D and degradation rate , 2 = D/. The uncharacteristically large  suggests that, within the framework of this simple model, either Bcd diffusivity or stability (or both) is enhanced in the large embryos. Since the impact of these changes on  is dampened by the square root function according to the simple diffusion model, the magnitude of such changes would have to be enormous in order to achieve the observed increase in . One observation that we made while evaluating our bcd mRNA FISH data was the relatively diffused mRNA distribution at the anterior. A diffused bcd mRNA would provide a plausible explanation for the observed Bcd gradient profiles in the large embryos (see Discussion).

Fig. X shows the raw epifluorescence images of the large and small embryos that have undergone FISH detecting bcd mRNA. In these figures, we also show the Otsu outline that marks the detection areas for specific signals (see Methods). A quantitative comparison between the large and small embryos revealed a significantly larger signal area in the large embryos (?? And ??, P= ??). To determine whether large embryos by default have significantly much larger signal areas for bcd mRNA than small embryos, we evaluated the Otsu outline areas in embryos from our previously published pair of inbred lines as well as pairs of embryos from population cages. Our results show that these signal areas are generally insensitive to embryo size. In cases where the signal area differences between large and small embryos reach statistical significance, the relative differences are always small (<10%?). This is in sharp contrast to the relative difference between the large and small embryos from the alternative lines that we study in this report, which exceeds 2 fold (p =??). These results document that the large embryos from the selected line have bcd mRNA distribution properties uncharacteristic of normal Drosophila melanogaster embryos.

**Discussion**

The current report extends our investigations of the mechanisms for Bcd gradient scaling within a species (ref). Our studies take advantage of Drosophila melanogaster inbred lines that were selected for the extremes of egg size (ref). While stochastic variations in egg size within an established lab line are small (ref), the egg size difference between the selected lines is greatly enhanced (L difference value? Volume difference?). This makes it possible to quantitatively interrogate the properties of the Bcd gradient to identify specific mechanisms for within-species Bcd gradient scaling. These inbred lines were derived from random crosses of the wildly collected flies. The selection process can thus be viewed as “reshuffling” of the naturally occurring genetic variations to yield desired traits, in this case, the extremes of egg size. The embryos from the alternate pair of inbred lines described in this report exhibit Bcd gradient properties uncharacteristic of their size. Specifically, the large embryos have lower B0 than small embryos. Despite the “reversed” B0 properties in these embryos, the hb expression boundary position is scaled with L. Importantly, the Bcd gradient profiles in these embryos are scaled with L: expressing the profiles as a function of relative AP position x/L reduces the differences in both the mean Bcd concentrations and Bcd-encoded mean positional information between the embryos (Fig X). Unlike the documented “normal” within-species scaling that is achieved by adjusting B0 (ref), the scaling in the embryos described in the current work is achieved by scaling  with L. Our report thus provides a first documentation that within-species Bcd gradient scaling can also be achieved, in a specific case that is uncharacteristic of normal Drosophila melanogaster (see below), through adjusting the exponential shape characteristics of the Bcd gradient profile, a mechanism employed by embryos from different species to achieve scaling (ref).

Our results suggest that the large embryos described in this report, not the small embryos, exhibit properties uncharacteristic of Drosophila melanogaster. In this species the Bcd gradient profiles have a length constant of ~100 m, a value that is constrained by the species-specific properties relevant to Bcd gradient formation, namely, the effective diffusion constant D and effective degradation rate  of Bcd in the early embryo. The large embryos that we study in this report have more maternally deposited bcd mRNA than the small embryos, suggesting that maternal deposition of bcd mRNA remains intact. Consistent with this notion is our observation that aggregate B detected in the large embryos remains higher than that in small embryos (see Methods). We found that the large embryos that we study here have a diffused distribution of bcd mRNA (Fig. X), a property that is not shared by other large embryos (Fig. X). It is well documented that bcd mRNA that is not localized to the anterior may be subject to translational inhibition by nanos (ref). Thus the diffused bcd mRNA distribution in the large embryos can readily explain both the uncharacteristically low B0 and the uncharacteristically large  of 144 m. Our results further suggest that the inbred large-egg line acquired compensating properties to counter the extreme of large egg size to allow the embryos to have a scaled Bcd gradient profile.

It has been proposed that, based on our recent finding that hb transcription is quickly shut down at early cycle 14, Bcd may be able to directly pass its scaling properties to the hb expression boundary near mid embryo (ref). If scaled patterning near mid embryo does have a “special meaning” in landscape of the AP patterning (ref), then Bcd scaling in this part of the embryo might be under more desirable than other parts of the embryo. In the large embryos that we study here, xhb near mid embryo is scaled with L and, furthermore, Bcd gradient scaling in this part of the embryo appears to be “at the expense” of the anterior by reducing B in that part of the embryo. These results also suggest that additional mechanisms, such as cross-regulatory and/or other maternal inputs, must play a role to compensate for this shortage in B at the anterior to achieve robust AP patterning along the entire AP length. Understanding the molecular details of scaled patterning remains an important scientific challenge, and the Drosophila AP patterning will continue to serve as a powerful paradigm for future mechanistic studies. We note that, since the Drosophila embryo does not grow in size, it represents an excellent system for investigating the scaling problem exclusively at the level of pattern specification without an entanglement with tissue/organ growth.

SAVED stuff for potential future use

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<<<Delete—incorporate In particular, describe all details as necessary–fill the blank (Spell out the line number in legend and Methods—maybe once in text): 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. >>>

**Alternate large embryos develop scaled patterning**

Despite the 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.

. 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.

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.

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.

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. -------------------------------------------------------------------------------------------------------

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.

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, 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 our deeper 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 additional mechanisms 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 oogenesi sprocess with regard to the egg volume-dependent maternal deposition of bcd mRNA to the egg.