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Natural variation in embryo mechanics: gastrulation in *Xenopus laevis* is highly robust to variation in tissue stiffness

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Abstract

How sensitive is morphogenesis to the mechanical properties of embryos? To estimate an upper bound on the sensitivity of early morphogenetic movements to tissue mechanical properties, we assessed natural variability in the apparent stiffness among gastrula-stage *Xenopus laevis* embryos. We adapted micro-aspiration methods to make repeated, non-destructive measurements of apparent tissue stiffness in whole embryos. Stiffness varied by close to a factor of 2 among embryos within a single clutch. Variation between clutches was of similar magnitude. On the other hand, the direction of change in stiffness over the course of gastrulation was the same in all embryos and in all clutches. Neither pH nor salinity – two environmental factors we predicted could affect variability in nature – affected tissue stiffness. Our results indicate that gastrulation in *X. laevis* is robust to at least two-fold variation in tissue stiffness.

Keywords

Developmental biomechanics; morphogenesis; gastrulation; cellular mechanics; phenotypic variation

Introduction

Morphogenesis involves the deformation and movement of embryonic tissues, and in any material, deformation and movement depend on both the forces applied to the material and the mechanical properties of the material. Hence, from basic physical principles we expect that morphogenesis depends on developmentally regulated tissue mechanical properties, just as it depends on developmentally regulated force production (Koehl, 1990; Davidson et al., 1995; Keller et al., 2003; Jakab et al., 2008). These mechanical properties include stiffness, which relates strain to applied stress, and viscosity, which relates the rate of strain to applied stress. In addition, numerous recent studies demonstrate that substrate stiffness affects cell movement, force generation, spreading, gene expression, differentiation, proliferation, or apoptosis (Pelham and Wang, 1997; Lo et al., 2000; Wang et al., 2000; Saez et al., 2005; Engler et al., 2006; Georges et al., 2006; Guo et al., 2006; Saez et al., 2007). Substrate stiffness also affects morphogenesis of cultured mammary gland and vascular tissues (Sieminski et al., 2004; Paszek et al., 2005). Therefore, tissue mechanical properties could influence embryonic morphogenesis by acting as passive physical constraints, through mechanosensory pathways, or both (Ingber, 2006; von Dassow and Davidson, 2007).

However, it is unknown whether embryonic development is actually sensitive to variation in tissue mechanical properties. Instead, there could be either passive or regulatory mechanisms that make morphogenesis in the embryo robust to variation in tissue mechanical

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properties (von Dassow and Davidson, 2007). By “robust” we mean capable of producing similar phenotypes despite variation in, or perturbations to, factors that affect the developmental process (von Dassow et al., 2000; de Visser et al., 2003; Hamdoun and Epel, 2007). Some hypothetical mechanisms of primary invagination during sea urchin gastrulation are highly robust to variation in stiffness, potentially operating over a stiffness range of several orders of magnitude (Davidson et al., 1995). Furthermore, the same proteins (actin, for example) that give cells their mechanical properties also contribute to force generation, so changes in force generation may be tightly linked to changes in tissue stiffness. This kind of link could – depending on whether it was positive or negative – either increase or decrease the sensitivity of morphogenesis to tissue mechanical properties. Finally, the mechanosensory processes mentioned above could allow cells to match the forces they produce to the stiffness of the tissue, so they generate the same degree of deformation independent of the tissue’s mechanical properties, as has been found in MDCK epithelial cells (Saez et al., 2005).

Determining the sensitivity of morphogenesis to tissue stiffness is central to understanding morphogenesis. If morphogenesis is sensitive to tissue mechanical properties, elucidating the molecular mechanisms that regulate those mechanical properties will be just as important for understanding development as elucidating the mechanisms that regulate cell shape change and movement in the embryo. On the other hand, if morphogenesis is robust to variation in tissue mechanical properties, elucidating the passive or regulatory mechanisms responsible for that robustness will be important.

For a number of reasons, addressing these questions requires assessment of natural intraspecific variation among embryos in tissue mechanical properties. First, natural variation is both the product of the evolved regulatory mechanisms that control development, and the context in which selection acts during the evolution of morphogenetic mechanisms. Development has evolved to proceed successfully in the face of those perturbations that organisms frequently encounter in their natural environment (Hamdoun and Epel, 2007), and environmental factors can also be important modulators of development (Gilbert, 2001). Changes in the process of gastrulation are unlikely to spread through a population if they lead to failure of gastrulation over a substantial fraction of the range of natural variation in embryo mechanical properties. However, there would be little advantage to changes that increase robustness to perturbations well beyond the normal range of variation of those mechanical properties.

Second, robustness to natural variability in tissue mechanics may not be equivalent to robustness to experimental perturbations in stiffness. Manipulative experiments are ideal for identifying genes or processes that have major effects such as *Xflop* (Tao et al., 2005) or f-actin polymerization and myosin II activity (Zhou et al., in prep). But natural variation in tissue mechanics may be the sum of small amounts of variation in several different pathways at once. Hence, robustness or sensitivity to variation in tissue mechanics driven by experimental manipulation of one or a few pathways may not be the same as robustness or sensitivity to tissue mechanics in nature. It is the sensitivity to natural variation that is most relevant to the evolution and ecology of development.

Finally, determining the level of embryo-to-embryo variation will be useful for designing and interpreting manipulative experiments to assess sensitivity of morphogenesis to tissue mechanical properties. The level of embryo-to-embryo variability in stiffness should affect the extent to which experimental perturbations of stiffness lead to morphogenetic defects. If natural variability is high, one would expect that there would be a high degree of variation in the morphogenetic effects of an experimental treatment that alters stiffness alone, no matter how uniformly the treatment is applied.

If developmental changes in stiffness are important in morphogenesis, they also have to be consistent from embryo to embryo and clutch to clutch. One of the few studies on tissue mechanical properties during vertebrate gastrulation found that the dorsal marginal zone stiffens in the anterior-posterior direction (Moore et al., 1995), but the reported variation in stiffness is sufficiently high that it is not clear whether all embryos showed such stiffening or not. To address the role of tissue mechanics in morphogenesis, and to understand the physical basis of morphogenesis, we need to ascertain the natural level of mechanical variability, and whether developmental patterns of stiffness are consistent from embryo to embryo.

Previous studies tend to indicate high variation in the mechanical properties of early embryos cultured under uniform conditions (Waddington, 1939; Moore et al., 1995; Davidson et al., 1999; Wiebe and Brodland, 2005), but they have not attempted to separate real biological variation from experimental error (von Dassow and Davidson, 2007). To separate embryo-to-embryo variation within a clutch from experimental noise, one has to have an experimental design in which multiple measurements are performed on the same embryo.

To address these fundamental issues, we have adapted the micro-aspiration method to work with gastrula stage *Xenopus laevis* embryos (Fig. 1) and used it to assess the magnitude of natural variation in tissue mechanical properties among embryos. Micro-aspiration methods use the length of material pulled into a channel by suction pressure to calculate the material's mechanical properties (e.g. Mitchison and Swann, 1954a;Boudou et al., 2006). During gastrulation the ectoderm spreads over the embryo as the endoderm and mesoderm internalize, so that the endoderm is surrounded by mesoderm, which is in turn surrounded by ectoderm (Fig. 1B–C). Gastrulation provides some of the first large-scale, accessible morphogenetic movements in the amphibian embryo.

Our implementation of micro-aspiration allows non-destructive measurement of tissue mechanical properties in frog embryos. Thus, we can (1) take multiple measurements of an individual embryo to assess variation in tissue stiffness among embryos and (2) observe subsequent development to determine whether morphogenesis proceeds normally. Because micro-aspiration is a low-tech but effective method for measuring tissue mechanics, it will be convenient for any molecular developmental biology lab.

Natural variation can be broken up into several components. Even in controlled laboratory conditions there can be great variation among embryos in the relative rates of different morphogenetic processes during amphibian gastrulation (Ewald et al., 2004; Scobeyeva, 2006). Therefore we assessed within-clutch and between-clutch variation in the stiffness of gastrula stage embryos reared under uniform conditions, considering only those embryos that successfully internalized their endoderm.

Environmental factors also contribute to natural variation in development (Gilbert, 2001; Hamdoun and Epel, 2007). Therefore we consider the contribution of two environmental factors – pH and salinity – that could be important in natural populations. *Xenopus laevis* inhabits ponds that vary greatly in pH (Picker, 1985) and in the concentration of dissolved minerals (Godfrey and Sanders, 2004). Both the concentration of individual ions (especially calcium) and the osmotic pressure could contribute to tissue stiffness. Osmolarity influences stiffness in sea urchin eggs (Mitchison and Swann, 1954b; Mitchison and Swann, 1955), chondrocytes (Guilak et al., 2002) and the frog notochord (Adams et al., 1990). In addition pH affects the stiffness of sea urchin oocytes (Mitchison, 1956), and left-right asymmetry in *Xenopus* (Adams et al., 2006).

We found that **(1)** there is substantial variation in the mechanical properties of embryos at small spatial and temporal scales; **(2)** the apparent tissue stiffness increases over the course of gastrulation; **(3)** this increase in apparent stiffness occurs consistently in all embryos; **(4)** there is approximately two-fold variation in apparent stiffness among embryos even within a uniform environment; but **(5)** neither the concentration of dissolved salts nor the pH had a detectable effect on the apparent stiffness. Our observations suggest that *Xenopus laevis* gastrulation is insensitive to substantial variation in the apparent stiffness, and that the apparent stiffness is not tightly regulated. *X. laevis* gastrulation is instead robust to *at least* two-fold variation in tissue stiffness.

Results

The embryo-sucker

Our micro-aspirator allowed clear views of the aspirated tissue (Fig. 1D–E) as well as of particles within the channel, which could be used to observe the presence or absence of flow in the channel. With an open channel (no embryo in place) we could detect flow driven by as little as 25 μL change in volume (0.03 Pa), and over the course of a 15 minute measurement period (at −5.4 Pa suction), the drift in the zero point was typically less than or equal to 100 μL (0.12 Pa). Hence, this apparatus allows fine control of the pressure over the desired range (≤ 10 Pa).

At time scales of several minutes, the apparent stiffness showed a response consistent with published models of viscoelastic materials (Sato et al., 1990), with a high instantaneous stiffness, declining over time towards a more-or-less steady value (Fig. 1F). As predicted by models for homogenous linear materials (Sato et al., 1990; Aoki et al., 1997; Boudou et al., 2006), suction at different pressures produced similar values of apparent stiffness (1F). In addition, the aspirated tissue returned to its initial position after removal of the pressure gradient indicating that there was little creep over the course of 13 minutes (Fig. 1G).

Apparent stiffness was calculated using an elastic half-space model (i.e. infinite tissue thickness); but the actual tissue has finite thickness, and the thickness changes from stage to stage, and possibly from embryo to embryo. To test whether our measurements of apparent stiffness are substantially affected by the thickness of the tissue, we measured the minimum tissue thickness in the dorsal quadrant between the blastopore and the equator of the embryo during the stages of blastopore closure. Embryos were fixed at three time points spanning most of the period of blastopore closure. Tissue thickness was >100 μm at all points within the region of interest for all measured embryos (73 embryos from 3 clutches). Using a model for analysis of micro-aspiration for finite thickness materials (Boudou et al., 2006) we found that finite tissue thickness should contribute ≤16 % error for any single measurement at the locations and stages in development investigated in this study. Hence, approximating the tissue as a half-space should not substantially affect our measurements.

With care not to over-compress or wound the embryos during manipulation, measurements of stiffness could be made without blocking gastrulation (blastopore closure and endoderm internalization). However, it should be noted that even mild compression sometimes forced a small mass (≤ 250 μm diameter) of endoderm out of the blastopore, which hung on outside the blastopore by a narrow tether (≤ 70 μm diameter). The embryos in which this occurred typically looked otherwise indistinguishable from normal embryos. When manipulated even gently, the mass of endoderm would fall off, revealing that the blastopore had closed almost entirely. Such embryos were counted as having closed their blastopore.

Small-scale mechanical variability

Over a time scale of minutes, aspirated embryonic tissues frequently show periods of contraction and relaxation (Fig. 2). To characterize the long time scale behavior of the tissues we observed the embryos for 15 minutes with no suction followed by 45 minutes with suction of −5.4 Pa. Measurements were done at a variety of positions including ventral, lateral, and dorsal marginal zone, and endoderm, but not animal cap where aspirated tissue would be thin relative to the diameter of the channel. Embryos were at late stage 9 to stage 10 (at the start of gastrulation) at the start of the 1 hr period of observation.

We observed at least four types of behaviors (Fig. 2A; Supplemental videos S1–4). In some cases neither contraction nor obvious relaxation was observed: the tissue behaved as expected for linear viscoelastic materials. In other cases rapid contractions or relaxations were observed. Often the contractions were followed quickly by relaxation. Finally, we occasionally observed long, slow periods of contraction. In some cases rapid contraction/relaxation events were observed, superimposed on slow retractions. In most cases in this experiment, these contractions/relaxations were not observed over the course of the first ten minutes of the experiment (Fig. 2B). During this time, the tissue behaved as expected for a viscoelastic material. However, in a few cases in subsequent experiments, contractions or relaxations were observed much earlier. Occasionally it appeared as though aspiration was begun while the tissue was contracted, and it relaxed at some point during suction (Fig. 2A). While it is possible that the slow retractions resulted from settling of the embryo or tissue movements during morphogenesis, two observations suggest that the rapid contractions are unlikely to be due to shifts in the embryo. First, they are often followed quickly by relaxations (Fig. 2A and C). Second, contractions occasionally initiated at, and spread from discrete sites on the tissue surface (Fig 2C; Supplemental video 3).

Time-lapse sequences of embryos that retained their vitelline membrane and were only gently held in place with clay suggest at least two possible sources of the contractions and relaxations we observed in our micro-aspiration experiments. First, during cell division, neighboring cells were stretched along the plane of the division (Fig. 2D and supplemental movie S5). Second, we observed occasional contractions of groups of cells in 25 % of embryos (n = 40) covering a region up to a few hundred micrometers in diameter (Fig. 2E; and supplementary movies S5–7). Aspiration of the tissue is likely to make contractions more apparent than they would be in un-manipulated embryos because partial aspiration changes the geometry over which the contraction happens, magnifying the effect of a contraction (Supplemental Model S1A).

A homogenous material would appear smooth and symmetrical following suction. In some cases the aspirated tissue appeared smooth, but in other cases it appeared lumpy or asymmetrical (Fig. 1D and E). The size and prominence of the bumps varied among embryos and changed over time both prior to and during suction. This observation suggests small spatial scale heterogeneity in tissue mechanical properties is present in *Xenopus* embryos.

Changes in stiffness during gastrulation

To investigate changes in stiffness over the course of gastrulation and within-clutch variation in embryo stiffness we compared pairs of embryos from each of several clutches collected on different days (“within-clutch experiment”). Five pairs of measurements were made on each of two randomly selected embryos from each clutch (one clutch per day). It proved difficult to precisely stage embryos after they were deformed during micro–aspiration, so data were recorded relative to the time of the start of gastrulation (start of stage 10). Stiffness measurements were made over the course of 5 to 6.5 hours: from the

start of gastrulation until blastopore closure was nearly completed (stage 12.5). To maintain the same rate of development from day to day, room temperature was maintained at 21.5° C. The order in which the two embryos were measured was randomized within each pair of measurements to reduce the potential for bias associated with changes in stiffness over time. Because our interest is the normal variation in embryo mechanics, only pairs of embryos in which both embryos successfully completed blastopore closure following measurement were used. In a single case an embryo failed to form neural folds, but blastopore closure was normal, so the embryo was included in the analysis. In total, we were able to make successful measurements of 14 embryos from 7 clutches.

The experiment described above allows assessment of variation among embryos within clutches, but possible day-to-day variation in the apparatus and operator – all of which constitute measurement error – may be conflated with real clutch-to-clutch differences among the embryos. To assess variation between clutches, we conducted a similar experiment, but instead of comparing pairs of individual embryos, we measured 5 embryos (one measurement per embryo) from each member of several pairs of clutches ("between-clutch experiment"). The clutches and embryos were selected and prepared as described above. Data were recorded relative to the time of the start of gastrulation as measured separately for each clutch. Measurements were done similarly to those in the within-clutch experiment, and the same criteria were used to restrict measurements to those embryos in which blastopore closure occurred successfully. In total, we obtained successful measurements for 40 embryos from 4 pairs of clutches.

The data from the two experiments overlapped over the whole range, indicating that repeated measurement of the same embryo in the within-clutch experiment does not strongly affect the average magnitude of the apparent stiffness (Fig. 3A, B). In both experiments, the displacement ("D450," measured 450 s after suction) appeared to decrease and apparent stiffness ("E450," measured 450 s after suction) increased non-linearly with time (Fig. 3A, B).

Furthermore, in both experiments the magnitude of variation in stiffness increased dramatically over time as stiffness increased (Fig. 3B: note the logarithmic scale). Therefore prior to analyses, we applied two transformations to the data that reduced the dependence of variance on time, and linearized the trend in the mean with time. Using two transformations allowed us to determine whether our results were strongly dependent on the specific mathematical forms of each transformation. These transformations were the following. First, we analyzed displacement at 450 seconds following the start of suction (D450) as a function of the natural log of time, Ln(t), from the start of gastrulation.

$$D450=a^{*}Ln(t)+b \tag{1}$$

Second, we analyzed the natural log of the apparent stiffness at 450 seconds, Ln(E450), as a function of time, t:

$$Ln(E450)=f^{*}t+g \tag{2}$$

Where a, b, f, and g are fitted parameters. To investigate whether the trend in stiffness with time was consistent from embryo to embryo, we calculated the slope ("a" or "f") of the least-squares regression lines of D450 vs Ln(t) or of Ln(E450) vs t for each embryo in the within-clutch experiment, and each clutch in the between-clutch experiment.

From these analyses we find that embryos consistently increase in stiffness over gastrulation (Fig. 3C–F): the slope of D450 versus Ln(t) was negative, and the slope of Ln(E450) versus time was positive in every embryo in the within-clutch experiment (n = 14) and in every clutch in the between-clutch experiment (n = 8). The decrease in D450 with Ln(t) was strongly significant (Table 1), as was the increase in Ln(E450) with time (Supplemental table S1). We noticed that the average slopes differed between the two experiments, but the confidence intervals for the average slope overlap, indicating that the difference was not statistically significant (Table 1 and supplementary table S1).

Embryo-to-embryo variation

In order to estimate embryo-to-embryo variation we carried out nested-ANOVA analyses (Sokal and Rohlf, 1994). In the within-clutch variation experiment, we made multiple measurements of stiffness on individual embryos to try to distinguish embryo-to-embryo variability from random measurement error. To account for the developmental change in stiffness over the course of gastrulation, we calculated residuals of displacement after accounting for the least-squares regression line of D450 vs Ln(t) through the whole data set for either the within-clutch or the between-clutch experiment. The residuals were then analyzed in a nested-ANOVA model to estimate variance components associated with different random factors, and confidence intervals for those variance components (Sahai and Ojeda, 2004b). For the within-clutch experiment, the factors considered were “Embryo” which is a random factor accounting for embryo-to-embryo differences within the clutch, and “Day+Clutch” which accounts for random clutch-to-clutch differences and day-to-day differences in the equipment and operator. Similarly, for the between-clutch experiment, the factors included in the model were “Clutch” which accounts for random variation among clutches, and “Day” which accounts for variation among days in the equipment and operator.

In the within-clutch experiment, embryo-to-embryo differences (the factor “Embryo”) contributed significantly to the measured variation in displacement (Table 2). In the between-clutch experiment, clutch-to-clutch differences (“Clutch”) contributed significantly to the variation in displacement (Table 2). Analyses of residuals of Ln-transformed stiffness versus time gave similar results, (supplemental table S2), however, the variance component associated with within-clutch differences among embryos in Ln-transformed stiffness was not quite significant ($P = 0.06$). The timing of the first measurement in each pair of embryos or of clutches did not affect the estimated variance components (Supplemental Tables S3 and S4).

Gastrulation occurs over 2-fold range of stiffness

Variance components for displacement and Ln-transformed stiffness are useful for statistical analysis, however they do not have a clear physical interpretation, so we used these variance components to calculate a ratio (R_D) of the stiffness of high-stiffness embryos to the stiffness of low-stiffness embryos. R_D represents the ratio of the stiffness of an embryo (or of a clutch in the Between-Clutch experiment) lying one standard deviation above the mean on the displacement scale to the stiffness of an embryo one standard deviation below the mean on the displacement scale:

$$R_D=(\boldsymbol{D_{450}}+(s^2)^{1/2})/(\boldsymbol{D_{450}}-(s^2)^{1/2}) \quad (3)$$

Where s^2 is the variance component associated with either the factor “Embryo” or “Clutch” and ***D450*** is the mean value of displacement, based on the regression line through all the data from the experiment, at 3 hours after the start of gastrulation. This time point was

chosen since it was the midpoint of the measurement period. A similar metric R_{lnE}, can be derived on the Ln-transformed stiffness scale, producing similar results (supplementary table S2). These metrics represent an estimate of the range of variation in stiffness.

Different embryos within a clutch can differ in stiffness from each other by as much as a factor of two in stiffness, and different clutches also can differ from each other by almost a factor of two. The variance components associated with embryo-to-embryo differences within-clutches gave a ratio of high- to low- stiffness, R_D, of 1.9; and the variance components associated with clutch-to-clutch differences in the Between-Clutch experiment gave a value of R_D of 1.7. Analyses of the residuals of Ln-transformed stiffness versus time gave similar estimates (Supplemental tables S2).

Only a minor fraction of the variation in embryo stiffness could be accounted for by variation in developmental rates among embryos or among clutches. There was little variation in developmental rates among clutches: co-cultured clutches in the between-clutch experiment typically began gastrulation within 15 minutes of each other. Furthermore, in the within-clutch experiment, the expected R_D given the observed within-clutch variation in the time of onset of gastrulation would be ≤ 1.2 (i.e. ≤ 20 % differences in stiffness).

We estimated the combined effect of within-clutch and between-clutch variation by adding the variance component estimate for the factor “Embryo” from the within-clutch experiment to the variance component for either the factor “Clutch” from the between-clutch experiment, or the factor “Day+Clutch” from the within-clutch experiment (which combines day-to-day and clutch-to-clutch differences). For the displacement data, these combined variance estimates (20.7 and 17.7 μm^2 respectively) give ratios of high-to-low stiffness embryos (R_D), including both within-clutch and between-clutch variation, of 2.4 and 2.1 respectively. Analyses of the residuals of Ln-transformed stiffness versus time produce similar results (Supplemental table S2). Hence, even under controlled laboratory conditions, we observed approximately two-fold differences in apparent stiffness among embryos at the time of gastrulation.

Effect of the environment on stiffness

In natural populations, development has to be robust to numerous environmental perturbations (Hamdoun and Epel, 2007). To begin to assess the extent to which environmental factors influence tissue mechanics in embryos, we compared the stiffness of embryos raised in high salt medium (1.3 × Modified Barth’s Solution [MBS]) to the stiffness of embryos raised in our standard culture medium (0.33 × MBS); and we compared the stiffness of embryos raised at pH 5.4 to embryos raised at pH 7.4 (our standard pH). We chose to investigate the effect of high salt medium because it is a standard method for inducing exogastrulation in amphibians, although it is known to be less effective in *Xenopus* than in some other amphibians (Sive et al., 2000). Comparing high salt medium to standard medium means we simultaneously altered the osmotic potential and the concentration of all the dissolved salts. *Xenopus* tolerates a wide range of pH in its natural habitats (Picker, 1985); however, in contrast to previous studies (Dunson and Connell, 1982; Picker et al., 1993), we were unable to reliably culture embryos below pH 5.4. Our goal in choosing these treatments was to use environmental perturbations just large enough to span the range over which gastrulation can occur.

Because we wanted to focus on effects on stiffness during gastrulation rather than on events before gastrulation, we put embryos into either high salt medium or into standard culture medium at stage 9. Similarly, for the pH experiment we put embryos into standard or low pH medium starting at stage 9. In contrast, the standard method for inducing exogastrulation involves rearing embryos in 1.3 × MBS from early blastula stages through gastrulation (Sive

et al., 2000). All embryos were measured at stage 10. Embryos were incubated at different temperatures (14.5° C to 21.5°C) so that they would reach the appropriate stage at different times. Because we measured stiffness of embryos cultured in either high salt or standard medium at different times for each clutch, we randomized whether the controls or the treated embryos were measured first for each clutch.

We did not detect effects of salinity or pH on tissue stiffness. For the Salinity and pH experiments, the data were analyzed with a 2-way crossed ANOVA. In these analyses, salinity or pH treatments were treated as fixed factors; clutch-to-clutch differences (factor "Clutch") and the interaction of clutch and the treatment (e.g. "Clutch*Salt") were treated as random factors. Data were analyzed as displacements (D450; Table 3) or Ln-transformed-stiffness (Ln(E450); supplemental table 5) as in the Within-Clutch and Between-Clutch experiments. Increasing salinity by a factor of 3.9 over our standard culture medium had no detectable effect on stiffness (Table 3; Fig. 4A), and at most an inconsistent, small effect on endoderm internalization (Fig. 4B). Large differences in pH also had no detectable effect on apparent stiffness (Table 3; Fig. 4C) and little or no effect on blastopore closure and endoderm internalization (Fig. 4D). Clutch-by-treatment interaction effects did not contribute to the variation in stiffness in either experiment (Table 3). We obtained similar results from analysis of Ln-transformed-stiffness (supplementary Table S5 and Fig. S2).

To test whether we could detect an effect of experimental perturbations on tissue stiffness, we treated embryos with a low concentration of Latrunculin B (0.3 μM), which de-polymerizes micro-filaments (Spector et al., 1999). These measurements were done with later stages than the salinity and pH experiments to ensure that any effect of Latrunculin B on the appearance of bottle cell contraction (Lee and Harland, 2007) would not affect the stage at which we conducted our measurements. Embryos were incubated at 21 to 22°C in either 0.3 μM Latrunculin B or a carrier control (0.006 % Dimethyl Sulfoxide [DMSO] medium) from the start of gastrulation (early st. 10). Stiffness measurements were made at 1 to 1.3 hours after putting the embryos into the Latrunculin B or DMSO medium. For this experiment embryos were not kept in agar wells after transfer to Latrunculin B or DMSO medium. We predicted Latrunculin B would soften the embryos and we wanted to reduce the chance that the maximum aspirated length would be greater than the radius of the channel, so we used lower suction pressures for the Latrunculin B experiment than for the other experiments. For the Latrunculin B *versus* carrier control experiment the baseline pressure was −0.85 Pa and the suction pressure was −3.6 Pa.

Latrunculin B reduced stiffness (increased D450) by about 19 %, a small but statistically significant amount (Table 3, Fig. 4E; Latrunculin B experiments were analyzed with 2-way crossed ANOVA as outlined above for pH and Salinity). At this concentration, Latrunculin B did not inhibit blastopore closure or endoderm internalization (Fig. 4F). Blastopore closure also occurred in many devitellinized embryos; however they had a tendency to dissociate starting at small nicks in the epithelium, making it impossible to determine the frequency of blastopore closure. We did not detect a significant clutch-by-treatment effect (Table 3). Similar results were obtained in analysis on the Ln-transformed stiffness scale (Supplementary Table S5).

Consistent with our observations in the Between-Clutch experiment, high clutch-to-clutch variation in stiffness was apparent in experiments testing pH, Salinity, or Latrunculin B (Fig. 4, Table 3; Supplementary Table S5 and Fig. S2). The same poly(dimethylsiloxane) (PDMS) channel was used throughout these three experiments, so this clutch-to-clutch variation is likely to reflect biological variation among clutches rather than variation in the equipment. In the Between-Clutch experiment day-to-day variation did not have a significant effect

(Table 2; Supplemental Table S2), also suggesting that clutch-to-clutch differences here (the pH, Salinity, and Latrunculin B experiments) reflect biological differences among clutches.

Discussion

During morphogenesis, the extent of cell or tissue shape change and movement depends on both the forces applied to the tissue and the mechanical properties of the tissue. Hence tissue mechanics is fundamental to understanding morphogenesis. To determine how sensitive morphogenesis is to tissue mechanical properties, we assessed the natural variability in the apparent stiffness of embryos. Natural variability in tissue mechanics places a minimum bound on how sensitive morphogenesis is to variation in tissue mechanical properties. This approach is complementary to studies on the molecular control of embryo mechanical properties (e.g. Tao et al., 2005; Zhou et al., in prep): such studies focus on only a few pathways at a time, whereas natural variation in stiffness may be the summation of small amounts of variation in many pathways at once. Furthermore, natural variation is the form of variation that is significant in the evolution of developmental processes.

Our findings indicate that gastrulation is robust to a two-fold or greater range of variation in the apparent tissue stiffness. We found that the direction of change in tissue stiffness during *X. laevis* gastrulation was the same in all tested embryos. However, the magnitude of the apparent stiffness varied greatly from embryo to embryo, and from clutch to clutch.

Gastrulation is robust to mechanical variation

We found that gastrulation occurred successfully across approximately a two-fold range of apparent stiffness. By making multiple measurements on individual embryos or individual clutches we were able to estimate the contributions of embryo-to-embryo variation within-clutches and between-clutch variability to the total variability in apparent stiffness. There was approximately 1.9 fold variation in stiffness among embryos within clutches, and approximately 1.7 fold variation in stiffness between clutches. Combining within-clutch and between-clutch variance estimates suggests that gastrulation can occur successfully over a 2.1 to 2.4 fold range in stiffness. The confidence limits for the variances components on which these ranges are based are very broad, but salinity, pH and Latrunculin B experiments also indicate high variability in stiffness among clutches. Even our lowest estimate for clutch-to-clutch differences in stiffness (from the Latrunculin B experiment) suggests that stiffness varies by 1.4 fold among clutches. These are conservative estimates of the range in stiffness since some embryos or clutches will fall beyond one standard deviation from the mean.

Environmental factors could contribute to variation in stiffness in natural populations. However, the two environmental factors that we tested, pH and salinity, had no detectable effect on tissue stiffness; whereas Latrunculin B reduced the apparent stiffness by almost 20 % but did not prevent blastopore closure. Latrunculin B is likely to also reduce force generation, but this has not yet been assessed.

Other studies on amphibian embryos have found very high levels of variability in tissue viscosity and stiffness (Moore et al., 1995; Wiebe and Brodland, 2005) or apparent surface tension (Davis et al., 1997); however these studies do not differentiate between real biological variability and experimental error. Few studies have investigated tissue mechanical properties in other organisms. One study on sea urchin embryos found high clutch-to-clutch variation in embryo stiffness (Davidson et al., 1999). Statistical analysis of the data from that study suggested that in sea urchins high-stiffness clutches were 30 to 70 % stiffer than low-stiffness clutches (von Dassow and Davidson, 2007). Hence, embryos

from two different phyla, the only two for which there are sufficient data, appear to show high levels of natural variability in stiffness.

While it appears that the stiffness of the embryo is not tightly regulated, it is worth considering that the stiffness of embryonic tissues is extremely low relative to other biological materials. For example adult mammalian “soft” tissues span more than 7 orders of magnitude in stiffness: from 17 Pa for fat to 3.1×10^8 Pa for tendon (Levental et al., 2007). Gastrula stage *X. laevis* embryos are at the lowest end of this scale (Fig. 3; Moore et al., 1995; Zhou et al., in prep). Furthermore, although the magnitude of the apparent stiffness was highly variable, we found that *X. laevis* embryos always became stiffer during gastrulation. This 2 to 6 fold increase in apparent stiffness over the course of blastopore closure (Fig. 3) was as large or larger than the variation in stiffness among embryos and among clutches.

Understanding the mechanisms that allow organisms to produce the same morphogenetic outcome despite widely varying physical properties will be very important for understanding morphogenesis, both at a mechanistic and evolutionary level. Several possible mechanisms could account for the observed robustness of morphogenesis to variation in tissue mechanics (reviewed in von Dassow and Davidson, 2007). First, the physical mechanism of morphogenesis may confer robustness over a broad range. For example, several hypothetical mechanisms for primary invagination during sea urchin gastrulation can produce an invagination over many orders of magnitude in stiffness, as long as the ratio of cell stiffness to extracellular matrix stiffness is above a threshold (Davidson et al., 1995). Second, the cytoskeleton is responsible for both giving a cell its mechanical properties and for generating forces and movement so it is possible that up-regulating cell stiffness directly leads to up-regulated force production and vice versa, thereby matching force production to tissue mechanical properties. Third, mechanosensory responses to tissue stiffness could allow cells to regulate force production to match the stiffness of their micro-environment. Such a mechanism would be consistent with the observation that adult cells in tissue culture studies often exert higher forces on stiffer substrates (Lo et al., 2000; Wang et al., 2000; Saez et al., 2005).

Small-scale temporal variation in tissue mechanics

The observed contractions and relaxations of the tissue imply that the tissue mechanical properties are variable at small temporal scales. Such small-scale temporal variability could be significant for processes such as cell movement and lamellipodia formation that occurs on similar time scales. We saw no obvious relation between the start of contractions or relaxations and the beginning of suction or compression of the embryo, so they are not likely to be triggered by manipulation of the embryo, but we cannot rule out the possibility that these manipulations affect their frequency. Previous authors have also observed transient contractions of large groups of cells associated with calcium waves in explants from the dorsal marginal zone, though not from other regions (Wallingford et al., 2001). It is noteworthy that tissue movements during amphibian neural tube closure and chick gastrulation appear to be pulsatile (Selman, 1958; Stern and Goodwin, 1977; Jacobson, 1978; Kucera and Burnand, 1987). Although we saw occasional contractions of groups of cells (Fig. 2, supplemental movies 5–7), we did not see any evidence that blastopore closure was pulsatile in *X. laevis*. Both contractions and relaxations of groups of cells, and cell divisions could produce the observed contractions in the micro-aspirator. In addition to the deformations associated with cell division, numerous studies demonstrate changes in stiffness during cell division (Mitchison and Swann, 1955; Sawai and Yoneda, 1974; Kunda et al., 2008).

Several authors have suggested that cellular responses to mechanical strain influence morphogenesis. Beloussov et al. (2006) suggested that cellular responses to strain are involved in the coordination of morphogenesis in amphibian embryos. Farge (2003) found that in *Drosophila melanogaster*, strain affected the expression of the gene Twist, which is involved in gastrulation. The rapid, localized strains that result from spontaneous contractions or cell divisions in the *Xenopus* embryo constitute biomechanical noise that any model of morphogenesis that involves strain-mediated cell behaviors must take into account.

Micro-aspiration as a tool in developmental biology

Micro-aspiration has several useful features for studying embryonic tissue mechanics. It can be applied at a range of spatial scales from individual cells (Tickle and Trinkaus, 1973; Sato et al., 1990; Hochmuth, 2000) to eggs (Mitchison and Swann, 1954a; Sawai and Yoneda, 1974; Nakamura and Hiramoto, 1978) and tissues (Gustafson and Wolpert, 1963; Ohashi et al., 2005). It can be done non-destructively so that multiple measurements can be made on individual embryos, and subsequent development can be assessed. Perhaps most importantly, it requires no specialized equipment, and the analysis is – with some caveats – straightforward, making it usable in any developmental biology lab. Micro-aspiration can also be adapted to look at a number of mechanical properties in addition to stiffness, including a material's Poisson ratio (Boudou et al., 2006), anisotropy (Ohashi et al., 2005), and viscosity (Sato et al., 1990; Zhou et al., 2005).

Our conclusions are unlikely to be strongly sensitive to our choice of mechanical model since we obtained similar results when we analyzed the data on a Ln-transformed stiffness scale (supplemental tables S2 and S5) as we did with the raw displacement data. The apparent stiffness is a measure of the stiffness of the bulk tissue that approximates the complex, cellular, layered, actively deforming material as a homogenous, linear material. This appears adequate for our present purposes (Fig. 1F), especially since the question we address in this study is not what the value of the stiffness is, but how variable the mechanical properties of the embryo are. However, this is only a starting point since it is clear that the tissue is not mechanically homogeneous (Fig. 1D) and it is actively contractile (Fig. 2).

Bulk mechanical properties of embryos are important because morphogenesis involves transmission of forces between distant parts of the embryo, and such transmission depends on tissue-scale mechanical properties. Blastopore closure in *Xenopus* appears to involve transmission of forces generated in the involuting marginal zone along arcs of tension that constrict the blastopore (Keller, 1984; Keller et al., 2003). Similarly in *Drosophila*, ablation of mid-dorsal parts of the embryo induce changes in deformations, morphogenesis, and gene expression at the anterior end of the embryo during gastrulation (Farge, 2003; Supatto et al., 2005); and in some species of sea urchins, applying suction to the lateral sides of the embryo can inhibit invagination of the archenteron at the vegetal pole during gastrulation (Takata and Kominami, 2001).

However, determining how signaling networks control large-scale tissue movements during morphogenesis will ultimately require understanding how regulation of cellular and sub-cellular properties, including contractility and architecture, controls the physical properties of tissues. For any complex structure, such as a limb held stiff by the action of muscles on tendons and bones, the stiffness of the structure as a whole depends on multiple factors. The apparent stiffness of the embryo aggregates contributions from material properties such as quantity of F-actin or other structural molecules, architectural features such as cells and tissue layers, and cellular contractility or pre-stresses within the tissue (Zamir and Taber, 2004a; Zamir and Taber, 2004b). Recognizing that morphogenesis occurs robustly despite high natural variation in the mechanical properties of the embryos will be crucial for

studying the molecular, cellular and tissue level factors that contribute to the mechanical properties of embryos and control the physical process of morphogenesis.

Further questions

We found that gastrulation in *Xenopus laevis* is robust over an approximately two fold or greater range of apparent stiffness. This suggests that work is needed to address three questions. First, what is the upper limit to the robustness of morphogenesis to variation in tissue mechanics? Second, what are the molecular and biomechanical mechanisms that allow gastrulation to be as robust to variation in tissue mechanics as it is? And finally, are other morphogenetic processes similarly insensitive to variation in the underlying mechanical properties of the tissues?

Experimental procedures

Micro-aspirator

Our micro-aspirator was constructed by casting PDMS around an optical fiber to form a channel in the PDMS. The optical fiber was prepared by stripping the outer two layers of protective coating from 2 to 3 cm of the fiber, leaving the glass fiber core exposed. PDMS elastomer base (Sylgard 184; Dow Corning Co., Midland, MI) was mixed with curing agent as per manufacturer's directions, poured into the mold around the fiber, and allowed to cure. Once cured, the optical fiber was removed and the PDMS block was cut with a razor blade mounted on a drill press to make a smooth, vertical cut, leaving a 120–125 μm diameter channel opening. The PDMS block was sealed between two 90 mm by 90 mm chambers with vacuum grease (Dow Corning, Co., Midland, MI). These chambers are a low-pressure reservoir and a high-pressure reservoir (Fig. 1A). We adjusted the pressure by adding or removing media from the low-pressure reservoir; and we calculated pressure differences by hydrostatics.

Between days of use, the apparatus was rinsed two to three times with distilled water, once with 70 % ethanol, and bathed in distilled water with one to four drops of 10 M NaOH. Before re-use, it was rinsed repeatedly in 0.33× MBS. The channel was cleaned as necessary with hair tools and suction, or with washes in 100 % ethanol and 4 M NaOH. The channels are sturdy, so they can be used repeatedly over several weeks as long as they are kept clean. Long-term use of a single channel is particularly valuable since the angle of the channel opening to the optical axis can have a significant effect on the apparent displacement (Supplemental Model S1B).

Embryo handling and media

Clutches of embryos (a group of eggs collected from the same female at the same time) were collected and fertilized following standard methods (Kay and Peng, 1991), and dejellied in 2 % Cysteine solution (pH ~8.3). In most experiments, embryos were dejellied at approximately 30 minutes after fertilization. Typically, 100 to 200 firm, round embryos that had rolled were selected and kept at 14.5° C until late blastula stages (st. 9). We used only clutches in which the majority of embryos rolled and appeared firm and round at the 1 cell stage, and the great majority of embryos were free of cleavage defects or other obvious defects at stage 9. Only embryos free of visible defects were retained for experiments. At stage 9, the vitelline membrane was removed with forceps, and the embryos were kept in small (1.6 mm diameter) hemispherical agar wells (1 % agarose) so that they would retain their spherical shape.

Embryos were staged according to the Nieuwkoop and Faber staging tables (Nieuwkoop and Faber, 1967). We defined the onset of gastrulation (start of stage 10) for a set of embryos as the time when half of the embryos showed a distinct line or patch of contracted bottle cells.

Embryos were cultured in 0.33 × MBS until late blastula stages (st. 9). Unless otherwise stated, the vitelline membranes were removed and measurements were done in our "standard" medium: 0.33 × MBS with 2 mg/mL bovine serum albumin ("BSA"; Sigma Aldrich), and 400 μL/50mL of antibiotic-antimycotic (A5955; Sigma-Aldrich, St. Louis, MO). BSA was added to reduce adhesion of the embryo to the measurement apparatus. In the within-clutch and between-clutch experiments, embryos were retained in this standard medium following measurements to observe subsequent development. In the Salinity and pH experiments the vitelline membranes were removed in the respective treatment media; and in the Salinity, pH and Latrunculin B experiments measurements were done in the respective treatment media. Standard medium was at pH 7.4. For the pH experiment, "Low pH" medium was the same as the standard medium but the pH was adjusted to pH 5.4 with HCl (measured with a Accumet AB15 pH meter; Fisher Scientific, Fair Lawn, NJ). High Salt medium was 1.3 × MBS with 2 mg/mL BSA, and 400 μL/50mL of antibiotic-antimycotic. 0.3 μM Latrunculin B medium and 0.006 % DMSO medium (as a carrier control) were similar to standard medium but with the addition of appropriate volumes of either 5mM Latrunculin B in DMSO or DMSO respectively.

We assessed the thickness of tissues in the micro-aspirated regions in whole embryos fixed in 4% paraformaldehyde, and bisected along a mid-sagittal plane with surgical scalpels. Images of the marginal zone were taken with a digital camera (Micropublisher 3.3, Q-Imaging Corp., Surrey, Canada) mounted on the camera port of a stereomicroscope (SZX12, Olympus, Melville, NY) with image acquisition software. For each clutch, embryos were fixed at 0.5, 3, and 5.5 hours following the start of gastrulation at 21° to 22° C. The minimum thickness of the tissue was measured as the smallest distance from the outer surface of the embryo to an internal space (either the archenteron or the blastocoel) at the region between the blastopore and the equator of the embryo on the dorsal side. Measurements were made with image processing software (ImageJ: Rasband, 1997 – 2008).

General test protocol

Unless otherwise stated, 8 to 16 randomly selected embryos, in which we had removed the vitelline membrane with little or no visible wounding, were selected and placed in agar wells. The embryos were monitored at 10 to 15 min intervals until they began to gastrulate, after which point embryos were selected at random for measurement. The remaining embryos were retained to monitor the success of blastopore closure and development for the clutch as a whole.

To make measurements an embryo was placed in the high-pressure reservoir at the opening of the channel in the PDMS and compressed just enough to form a seal on the opening. The embryo was placed in a holder consisting of two parallel PDMS strips sealed onto a glass cover slip with vacuum grease. A dissecting microscope was mounted above the micro-aspirator to allow manipulation of the embryo. The embryo was compressed onto the opening of the channel with a glass rod mounted on a micromanipulator, and fire-polished to a blunt, smooth tip. Unless otherwise stated a small baseline pressure drop (−1.2 Pa) was applied to test the seal and to improve the pre-suction image of the edge of the aspirated tissue (Supplemental Model S1C). After 5 minutes (or 15 for the experiments on long-time scale behavior, and on creep and linearity), a volume of water was removed from the low-pressure reservoir to apply suction to the tissue. This additional "suction pressure" was −5.4 Pa unless otherwise noted. The pressure was zeroed to within ± 0.06 Pa at the start of each measurement, and the system was checked for drift at the end of each measurement by

observing how much the volume of medium had to be adjusted to stop flow through the channel after removing the embryo.

Unless otherwise stated, measurements were made in the region on the dorsal quadrant between the blastopore and a position midway from the vegetal to the animal pole (Fig. 1B–C). All measurements were made at 21–22°C.

We assessed blastopore closure and endoderm internalization between late neural fold stages (st. 17) and the end of neural tube closure (st. 21). As much as possible, embryos from the same clutch were assessed at the same stage. A defect was recorded if a mass of endoderm ≥ 300 μm in diameter remained outside the embryo or if the blastopore was still open at least as wide as is typical for stage 12.5. In a few cases a small mass of endoderm remained outside of the blastopore, attached by a narrow (< 100 μm diameter) tether, but blastopore closure looked otherwise normal: the embryos were indistinguishable from un-measured but devitellinized embryos from their cohort after the mass of endoderm fell off. These embryos were counted as having successfully completed blastopore closure.

Imaging and analysis

To measure tissue deformation during micro-aspiration we visualized the opening of the channel with a 20× objective in an inverted microscope (Leica Microsystems, Bannockburn IL). A small amount of Sumi ink (Yasutomo, San Francisco, CA) was added to the medium so that flow indicative of leakage could be tracked within the channel. Time-lapse sequences were collected at 3 or 6 seconds per frame using a digital CCD camera (Scion Corp., Frederick MD) controlled by image acquisition software (ImageJ: Rasband, 1997 – 2008) and captured. Micro-aspiration time-lapse sequences were analyzed either by hand or with a custom macro in ImageJ that tracked the tissue surface movements.

To calculate the apparent stiffness from the amount of tissue aspirated we approximated the aspirated tissue as a uniform, incompressible, linearly elastic material with infinite thickness (e.g. the elastic half-space model Aoki et al., 1997; Boudou et al., 2006). Tissue thickness has little effect on the measured elastic modulus until the tissue becomes thinner than the diameter of the channel (Aoki et al., 1997; Boudou et al., 2006). In this simplified case, displacement into the channel following suction is inversely proportional to stiffness:

$$E = k^{*}\Delta P^{*}(r_c/D) \quad (4)$$

Where ‘E’ is the Young’s modulus in pascals, ‘ΔP’ is the suction pressure in pascals, ‘k’ is a constant (0.97), ‘r_c’ is the channel radius, and ‘D’ is the displacement: the distance between the pre- and post-suction positions of the tissue. The assumption of incompressibility implies that the Poisson ratio was 0.5. The Poisson ratio of the material can affect the displacement, but the effect would be at most 25 % if the Poisson ratio was actually equal to 0 (Boudou et al., 2006), the lowest possible value for normal materials.

Embryonic tissue acts like a viscoelastic material (Moore et al., 1995), but because of tissue contractions and relaxations, discussed above, it is difficult to make meaningful estimates of parameters for the viscoelastic model. Therefore, we analyzed displacement or apparent stiffness at 450 seconds after starting suction (E450). We only used measurements in which there was no visible leakage through the channel throughout the 450 s measurement period.

Statistics

ANOVA methods allow estimation of the contribution of different random factors (e.g. embryo-to-embryo, clutch-to-clutch or day-to-day differences) to the total variance of

measurements, as well testing for effects of fixed, experimentally controlled factors (Sokal and Rohlf, 1994). Experiments with nested factors (within-clutch and between-clutch experiments) were analyzed using nested ANOVA methods (Hernandez et al., 1992; Sahai and Ojeda, 2004b; Sahai and Ojeda, 2004a). Confidence intervals presented for variance components are the 90 % confidence intervals, since those correspond to a *P*-value of 0.05 for the one-sided test of whether the variance component is greater than zero (variances are by definition greater than or equal to zero: Sokal and Rohlf, 1994).

Methods for analysis of nested ANOVA's, and calculation of confidence intervals were implemented in a spreadsheet (Microsoft, Redmond WA). Statistical analyses of two-way crossed classification ANOVA designs (salinity, pH and Latrunculin B experiments) were calculated with statistical software (SPSS, Chicago IL). A fundamental assumption of ANOVA is that the variance is the same for all levels of each factor (Sokal and Rohlf, 1994), so we used data transformations as appropriate (when heterogeneity was obvious by visual inspection) to reduce this heterogeneity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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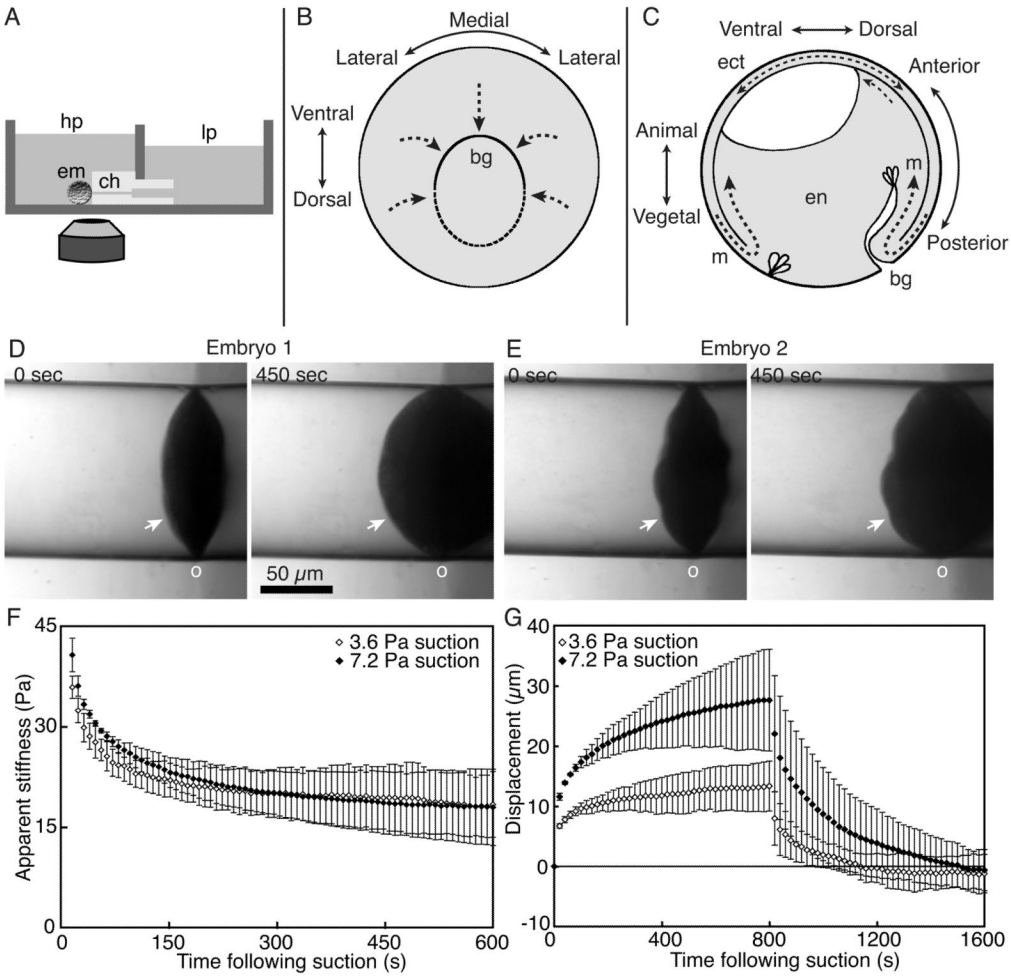

Figure 1.

(A) Micro-aspiration apparatus: an embryo (em) was pressed against a channel (ch) cast in PDMS connecting the high-pressure (hp) and low-pressure (lp) reservoirs. Tissue displacement into the channel was viewed through a microscope objective below. Pressure was adjusted by adding or removing media from the low-pressure reservoir. (B–C) Diagrams of gastrula stage embryos in vegetal view (B) and in cross section (C): arrows indicate directions of tissue movement; bg: blastopore groove; ect: animal cap ectoderm; m: deep-layer mesoderm; en: endoderm. (D–E) Representative embryos before and after application of −5.4 Pa suction showing smooth (D) or lumpy (E) appearance of tissue (arrows). The bulk of the embryo was to the right of the channel opening (o), but obscured by reflection off the PDMS surface. (F–G) Apparent stiffness (F) and displacement (G) versus time following suction at two different pressures: suction was ended at 800 seconds. Embryos in F and G were aspirated on the ventral side between the end of blastula stages (late st. 9) and early gastrula stage (st. 10.5). Baseline pressure was −2.4 Pa. Error bars indicate ±1 standard deviation (n = 5 embryos for −3.6 Pa, and n = 6 for −7.2 Pa). A, B and C modified from von Dassow and Davidson (2007).

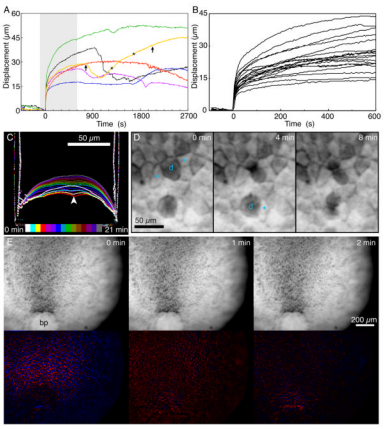

Figure 2.

Contraction and relaxation behavior. (A) Displacement versus time following suction (−5.4 Pa) for 6 embryos showing the variety of contraction and relaxation behaviors observed. Arrows indicate beginning and end of the period shown in panel C for the embryo shown in the gold curve. Note that for this embryo some leakage occurred following the contraction (between asterisks). (B) Displacement during the first 10 minutes (gray region in panel A) following suction (−5.4 Pa) in 19 embryos. Embryos in panels A and B were at the end of blastula stages (late st. 9) or beginning of gastrulation (st. 10) at the start of measurement. (C) The edge of partially aspirated tissue (colored lines) in an embryo undergoing a localized contraction while under suction. Arrowhead indicates site of initiation of contraction; the color bar (bottom) indicates the times associated with each colored curve; and the vertical bars are artifacts from the channel wall. Images from a time-lapse video were processed to outline the boundary of the tissue, and false color values were assigned to the different, superimposed frames. (D) Time series showing strain in cells (*) adjacent to cells undergoing division (d). Images were centered on the dividing cells by eye, and processed to enhance contrast and reduce noise. (E) An embryo in which most of the dorsal marginal zone underwent a rapid contraction. Top series: a sequence of frames from a time-lapse video (contrast enhanced, and image smoothed to reduce noise). Bottom series: Difference images between frames taken 1 minute apart show regions undergoing displacement from minute to minute. Blue: increase in brightness; red: decrease in brightness. Between minutes 0 and 1, a large region on the dorsal side contracted towards a point just above the blastopore (bp); little movement occurred between minutes 1 and 2; and a small amount of relaxation occurred near the blastopore between minutes 2 and 3. This embryo was at late blastopore closure stages (st. 12).

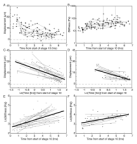

Figure 3.
Variation in apparent stiffness and displacement (at 450 seconds following suction) during gastrulation. (A) Displacement (D450) versus Time and (B) Stiffness (E450) versus Time for the between-clutch (filled symbols) and within-clutch (open symbols) experiments. Note in a single measurement, there was a strong contraction resulting in negative displacement, hence an infinite apparent stiffness. (C–D) Variation in D450 versus Ln-transformed time in the Within-Clutch experiment (C) and in the Between-Clutch experiment (D). (E–F) Variation in Ln-transformed stiffness versus Time in the Within-Clutch experiment (E) and in the Between-Clutch experiment (F). Best-fit lines for each embryo (C and E) or clutch (D and F) are shown (thin lines), as well as the best-fit line for the whole data set for each experiment (thick lines). Measurements were made in the region on the dorsal quadrant between the blastopore and a position midway from the vegetal to the animal pole (Fig. 1B–C).

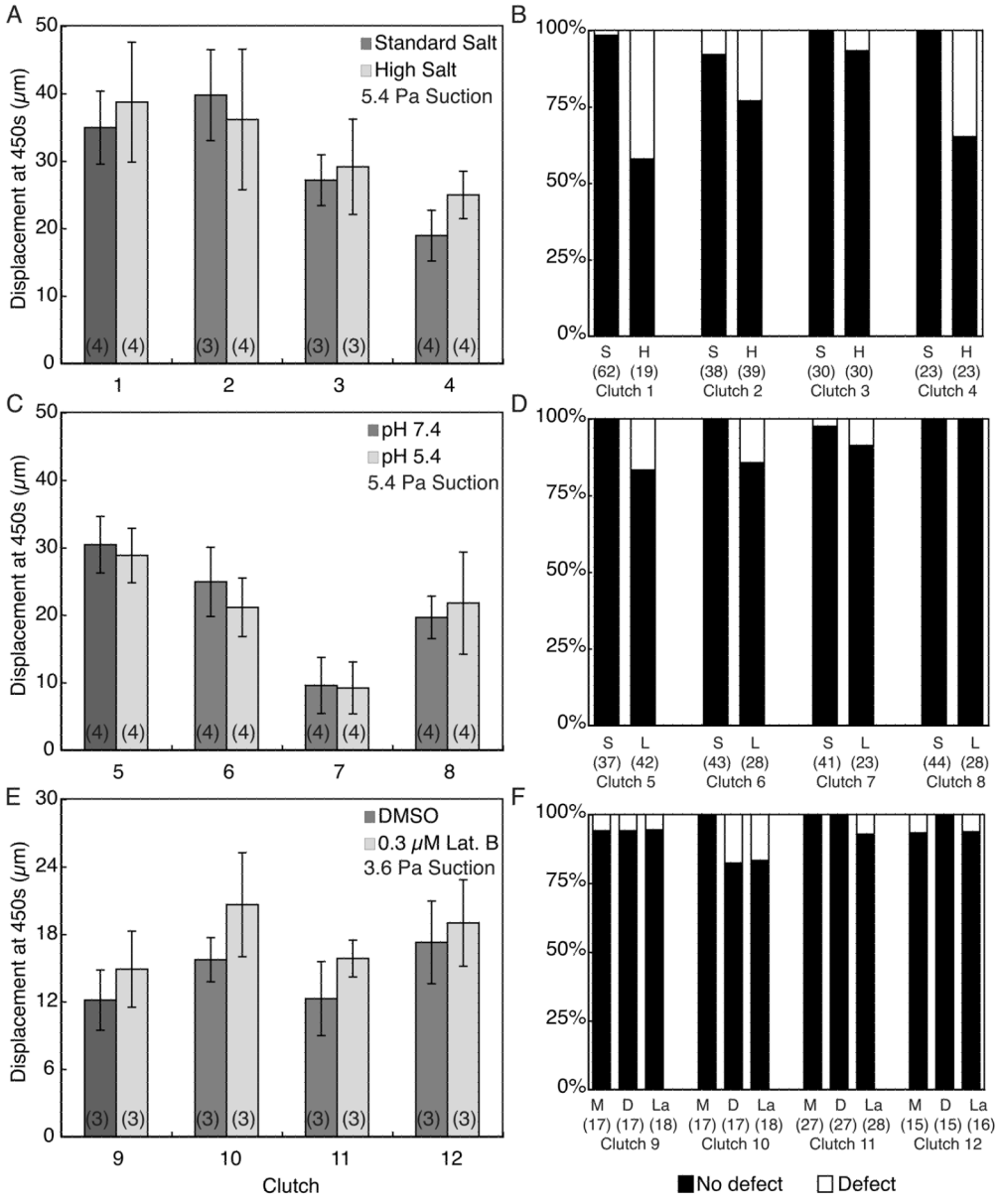

Figure 4.

Environmental and drug effects on apparent stiffness and success of gastrulation. (A, C, and E) Effects of treatments on displacement at 450 s following suction. Data were separated by clutch. Error bars indicate ±1 standard deviation. Measurements were made in the region on the dorsal quadrant between the blastopore and a position midway from the vegetal to the animal pole (Fig. 1B–C). (B, D, and F) Frequency of gastrulation defects (failure to close blastopore or failure to internalize endoderm) assessed between late neural fold and post-neural tube closure stages (st. 17 to 21, respectively). Sample sizes are in parentheses. S: standard salt and pH media (0.33 × MBS + BSA, pH 7.4); H: high salt media (1.3 × MBS +BSA, pH 7.4); L: low pH media (0.33 × MBS + BSA, pH 5.4); M: culture media (0.33 MBS); D: DMSO control (0.006 % DMSO in 0.33 × MBS + BSA); and La: 0.3 μM Latrunculin B, in 0.33 × MBS + BSA).

Table 1

ANOVA table for slopes of displacement (***D450***) versus Ln-Time

	Factor	df*	SS*	F	*P*-value	Mean Slope (Lower, Upper 95 % Confidence Intervals)** µm
Within-Clutch Experiment	Intercept	1	1093.1	27.8	**0.002***	**−8.8 (−12.9, −4.7)**
	Day+Clutch	6	236.04	2.83	0.1	
	Error	7	97.144			
Between-Clutch Experiment	Intercept	1	196.32	57.5	**0.005***	**−5.0 (−7.0, −2.9)**
	Day	3	10.242	0.172	0.9	
	Error	4	79.486			

‘SS’, Sum of Squares; ‘df’, degrees of freedom; ‘F’, sample F-statistic

* Significant at $P \leq 0.05$

** Confidence intervals calculated following Sahai and Ojeda (2004a).

Table 2

ANOVA table for variance components for residuals of displacement (D450) versus Ln-Time.

	Factor	df	SS	F	*P*-value	Variance Components[!!]: s^2 (Lower, Upper 90 % Confidence Intervals) μm^2	Ratio of high/low stiffness R_D
Within-Clutch Experiment	Day+Clutch	6	799.06	1.38	0.3	3.68 (0[!!], 39.0)	1.38**
	Embryo	7	674.62	3.69	**0.002***	**14.0 (4.07, 56.9)**	**1.90****
	Error	56	1463.9			26.1 (19.7, 36.8)	
Between-Clutch Experiment	Day	3	146.46	0.929	0.5	0[!!] (0[!!], 35.8)	—
	Clutch	4	210.25	2.73	**0.046***	**6.66 (0.106, 55.2)**	**1.66****
	Error	32	616.50			19.3 (13.3, 30.7)	

'SS', Sum of Squares; 'df', degrees of freedom; 'F', sample F-statistic

*Significant at $P \leq 0.05$

[!!]Negative variance component estimates were assigned a value of 0 since variance components are ≥ 0 by definition (Sokal and Rohlf, 1994).

**Mean D450 at 3 hrs used to calculate R_D were 12.1 μm (within-clutch experiment) and 10.3 μm (between-clutch experiment). Mean D450s were calculated using the regression line for all the data from either the within-clutch or between-clutch experiments.

Table 3

ANOVAs tables, D450.

Experiment	Factor	df	SS	F	*P*-value	Variance Components[!!]: s^2 μm^2	Ratio of high/low stiffness R_D
Salinity	Salt	1	29.847	0.965	0.4	—	—
	Clutch	3	1305.0	14.1	**0.03**[*]	**56.3**	**1.64**[**]
	Clutch[*] Salt	3	92.379	0.683	0.6	0[!!]	—
	Error	21	946.92			45.1	—
pH	pH	1	6.5568	0.544	0.5	—	—
	Clutch	3	1703.9	47.2	**0.005**[*]	**69.5**	**2.35**[**]
	Clutch[*] pH	3	36.135	0.541	0.7	0[!!]	—
	Error	24	534.11			22.3	—
Latrunculin B (LB)	LB	1	62.740	23.3	**0.02**[*]	—	—
	Clutch	3	115.69	14.3	**0.03**[*]	**5.98**	**1.36**[**]
	Clutch[*] LB	3	8.0632	0.251	0.9	0[!!]	—
	Error	16	171.10			10.7	—

'SS', Sum of Squares; 'df', degrees of freedom; 'F', sample F-statistic

[*] Significant at $P \leq 0.05$

[!!] Negative variance component estimates were assigned a value of 0 since variance components are ≥ 0 by definition (Sokal and Rohlf, 1994)

[**] Mean value of D450 for calculation of R_D: Salinity experiment: 31.1; pH experiment: 20.7; and Latrunculin B experiment: 15.9 μm. Mean D450's were calculated as the mean of all data in each experiment.