Extraembryonic assistance and stress: computational and experimental investigations of new developmental mechanisms.

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ABSTRACT

A great deal remains unknown about the mechanisms that underlie morphogenesis. Both endogenous pathways and external cues have an important role in the control of growth and form. In many species, organismal density is a contributing factor to morphological changes. Morphological remodeling based on the population of conspecifics in ones environment is seen in several species of fish, insects such as locusts, flatworms, and many other animals. Moreover, the phenomena of "stress buffering" shows that existing in a collective dampens individual response to stressors. In this study, we investigate the role of conspecific density on morphogenesis by observing how groups of *Xenopus laevis* embryos vs isolated embryos fare in response to developmental stressors, such as chemical teratogen and mRNA microinjections. This reframes morphogenesis as emergent behavior, not only at the level of cellular networks, but also in groups of complex organisms. Our hypothesis of morphogenetic assistance against stressors in conspecific collectives provides a fascinating basis for a computational approach; thus, we propose an ECA model of such phenomena to accompany our experiments. Finally, we hope to explore the role of endogenous stress signals during morphogenesis. Several studies have shown upregulation of stress biomarkers during natural developmental transitions and during regeneration. We theorize that stress is a driving force that guides developing organisms from one stage to the next, and not being at the correct stage at a given time point is a source of endogenous stress. In other words, we hypothesize that straying from the target morphology at a given time point is a source of "stress." To test this hypothesis, we investigate the stress marker profiles of embryos with morphological abnormalities.

INTRODUCTION

I. The influence of conspecific organismal density on morphogenesis

Morphogenesis

Morphogenesis is the set of remarkable processes in which a single cell gives rise to a complex organism with working anatomical structures that obey its species-specific laws of patterning. Investigating the governing processes of morphogenesis over development and regeneration is crucial to our understanding of life. How do networks of cells get the necessary information to achieve their target morphology and halt further growth at this stage? What instructive signals are transferred between local cells to create this global outcome? Most commonly, developmental biologists address these questions by studying gene regulatory networks: the genes involved in development and the transcriptional circuits that control their expression. Advances in molecular genetics over the past century have allowed for fascinating insights into the changes in gene expression that drive transitions to key stages of development.

While significant progress has been made in understanding the endogenous mechanisms that underlie the control of growth and form, it is now known that there are many external cues that influence development as well. Abiotic factors, such as temperature, magnetic fields, electricity, light, and chemicals, and biotic factors such as conspecifics, predators, and symbiotic relationships affect

morphogenesis. Organismal density has been observed to affect morphological outcomes across many species (1).

Density-dependent development

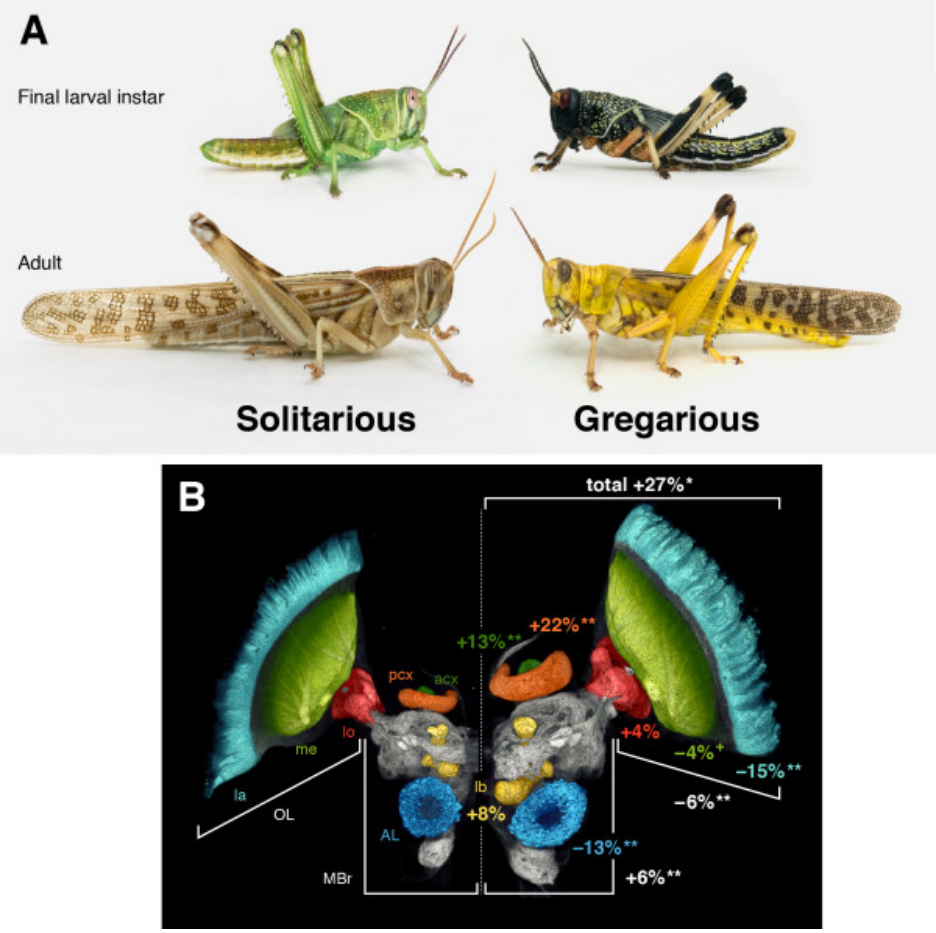


Figure 1: Solitatious vs gregarious locusts have differing morphological traits in both their juvenile and adult stages. A) Solitarious locusts have slightly larger bodies and colors that are more discreet, more suitable for camouflage, while gregarious locusts are smaller and have brighter colors such as yellow. B) Gregarious locusts have larger brains with differing morphology than their solitary counterparts. Reproduced from Burrows et al. 2011.

Density-dependent phenotypic plasticity is observed in species that have gregarious and solitary phases. Desert locusts, for example, exhibit a myriad of

morphological changes when transitioning from one of these lifestyles to another: solitary locusts are often green or brown, allowing for easy camouflage, while gregarious locusts can be bright red, orange, and yellow. Gregarious locusts also have smaller bodies than their solitary counterparts (2). Moreover, gregarious locusts and solitary locusts were found to have different brain morphologies: specifically, gregarious locusts have larger brain regions that are associated with higher integration (3, 4). This finding correlates with the wider diet of gregarious locusts in comparison to solitary locusts, which have much more restrictive dietary preferences. The morphological changes are accompanied by significant behavioral changes; while solitary locusts are only active during dusk and dawn and remain largely concealed, gregarious locusts form massive swarms that cover hundreds of kilometers squared (4). The oriental armyworm, *Mythimna separata*, also displays gregarious and solitary phases depending on crowding. Worms in crowds are smaller in size, darker in body color, have enhanced biopesticide resistance, enhanced digestive capacities, and were shown to have increased resistance to fungal infection. Additionally, crowded larvae showed increased developmental rates (5).

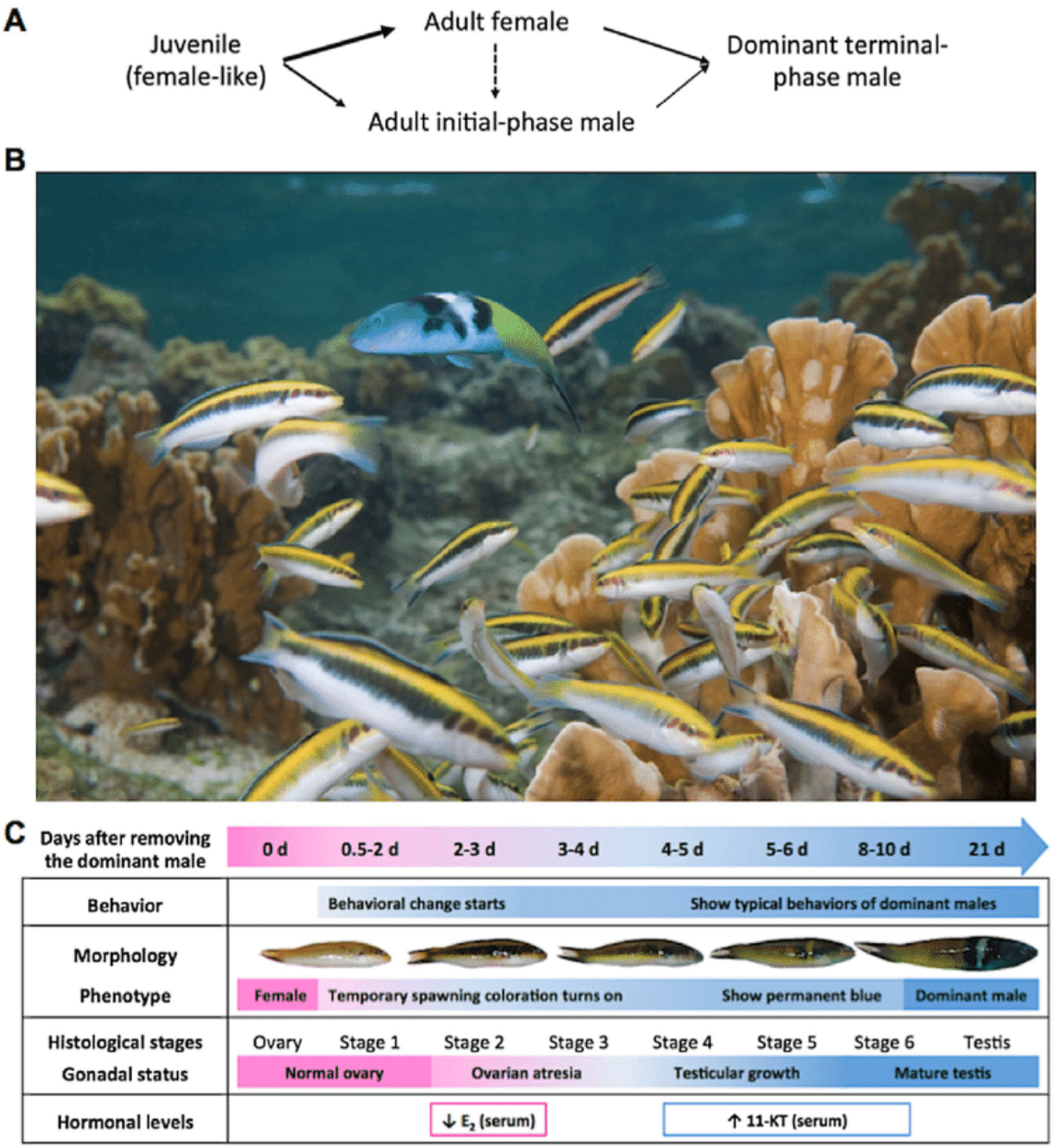


Figure 2. Bluehead wrasse are protogynous tropical reef fish. They have a polygynous social hierarchy, in which a dominant male has several female mating partners. Upon removal of the dominant male, the largest female in the group undergoes a female-to-male sex change, which involves significant morphological remodeling. Reproduced from Liu et al. 2016.

Several species exhibit morphological plasticity dependent on sex density. Sequential hermaphroditism, the process by which an organism changes its sex via remodeling of genital structures and regulating gene expression accordingly, is

seen in many species of fish and gastropods. Protandry is a system by which organisms develop as males and can later undergo a sex change to become female. Protogyny, a female to male sex change, is seen in polygynous networks in which a one male has several female mating partners (6). *Crepidula coquimbensi,* a marine gastropod species are protandric; the sex change is triggered in males that are less competent in finding a female mate during breeding season (7). Bluehead wrasse, a species of tropical reef fish, on the other hand, are protogynous. Most eggs develop as females, but a few are able to develop into dominant males. In the event of the dominant male's death, the largest female will undergo a sex change (6, 8).

Population density can also alter the course of development from larval to juvenile stages. *C. elegans*, a nematode species, can enter developmental arrest under unfavorable environmental conditions (9). It was shown that, following starvation, genes related to metabolism remained highly expressed in worms from low-density groups in developmental arrest, while worms from high-density arrested groups have repressed these genes. Conditioned media from high-density groups was enough to repress the genes in worms from low-density groups, suggesting the presence of a density-dependent soluble signaling molecule (10) . This indicates that higher conspecific density is significantly favorable during adaptation to stressful environmental conditions over the course of development.

The “stress buffering” phenomena

In many of the aforementioned examples, stressful conditions are posed, such as predation, harmful chemicals, the lack of a mate, starvation, *et cetera*. In various species, a phenomenon deemed “stress buffering” has been observed: the dampening of a stress response in a group of conspecifics (11, 12). In juvenile lake sturgeon, a cohesive social species, fish in a group had a shorter duration of cortisol response after a brief exposure to a stressor compared to isolated fish. Secondary stress markers from the plasma supported the hypothesis of a reduced stress response in groups (13). A similar study was conducted with zebrafish: fish exposed to alarm substances had a lower fear response in the presence of conspecific cues than when they were alone (14). The fields of psychobiology and neuroendocrinology have also extensively investigated this phenomenon. Studies of the hypothalamic-pituitary-adrenal axis, the endocrine subsystem responsible for stress hormone release and regulation, have confirmed that the assistance of a conspecific shortens and lowers the intensity of a stress response in many species, including rats, mice, hamsters, and primates from infancy through adulthood. Parallel studies in humans have reflected these findings. Psychological studies in humans have also shown that social support drastically improves response during traumatic situations (11, 15, 16, 17, 18).

Xenopus laevis as a model organism

Xenopus laevis, the African clawed frog, is an extensively studied organism, especially in the field of developmental biology. *Xenopus* embryos develop externally, lending themselves to easy observation and manipulation. Moreover, they develop rapidly in early (fertilization to tadpole) stages, allowing for convenience during experimentation. During artificial fertilization, mature frogs yield thousands of fertilized embryos, thus ideal for high-throughput experiments (19, 20).

The developmental stages of this organism have been thoroughly studied by developmental biologists for decades. Nieuwkoop and Faber cataloged a 66 defining stages of Xenopus development from fertilization to metamorphosis which is the standard for developmental biologists (21). Moreover, due to the breadth of developmental genetics studies conducted with this organism, there is a breadth of information about the gene regulatory networks that govern its development. However, the role of conspecifics during development has rarely been investigated. Given that amphibians lay very large clutches of eggs that develop in tandem, and that tadpoles of many frog/toad species tend to swim in dense aggregates in nature (22), this social cohesiveness may have importance during early development.

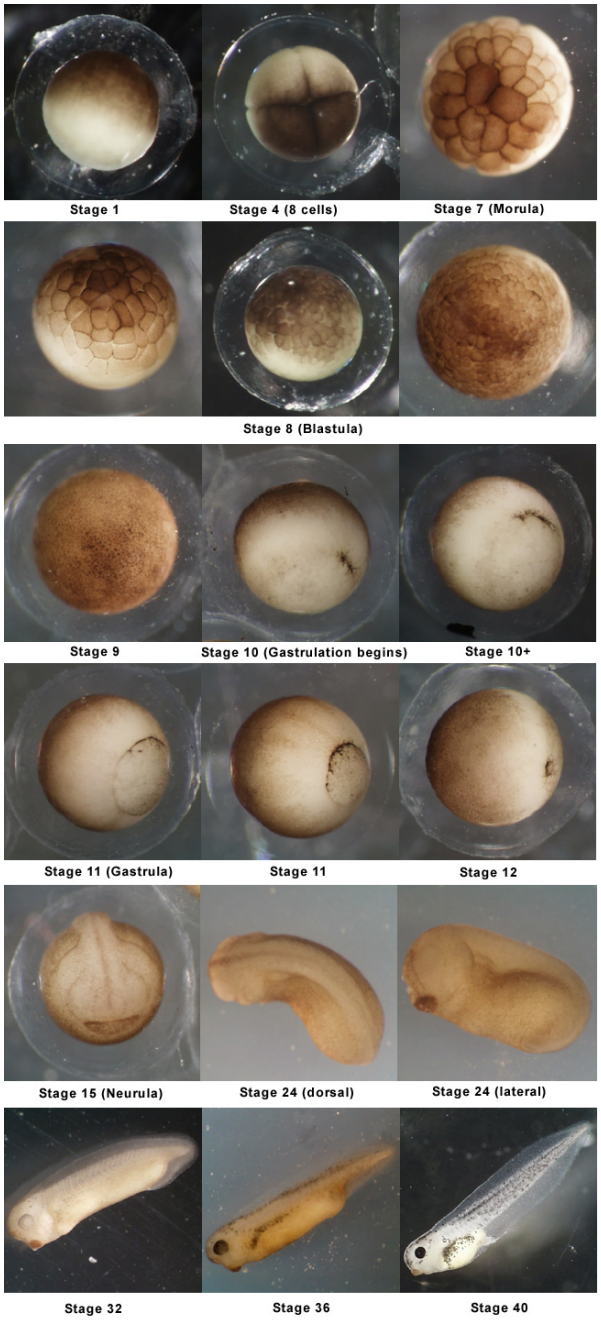


Figure 3. Xenopus laevis embryonic development, staged according to the Nieuwkoop and Faber chronology. Embryos begin at the one-cell stage, undergo cleavage, gastrulation, neurulation, formation of a tailbud, and then enter the pre-metamorphic tadpole stage. Reproduced from Swarthmore Developmental Biology Laboratory website.

Objectives and hypothesis

Many examples in nature support the hypothesis that there is safety in numbers; being in a group confers protection against various stressors. Moreover, changes in conspecific population density are known to influence early development and trigger morphological remodeling in many species. We seek to understand how developmental stressors will affect morphogenesis during embryonic development in individual *Xenopus* embryos in comparison with group-reared embryos. We hypothesize that embryos developing in a group will exhibit higher resistance to the effects of teratogen exposure. This will provide further insight into the role of external cues in morphogenesis.

To test this hypothesis, we will observe the effects of conspecific presence during exposure to a chemical teratogen as well as an mRNA microinjection.

We chose thioridazine, a commercially available antipsychotic drug used for treatment of schizophrenia and depressive disorders (23). It is a dopamine receptor antagonist which affects the development of early pre-metamorphic *Xenopus laevis*. Exposure to it during the neurulation stages has shown to cause significant morphological abnormalities, specifically in the craniofacial region (24).

Microinjection of synthetic RNA into *Xenopus* embryos is a widely used technique to study the effect of overexpression, gain of function mutations, and loss of function mutations (62, 63). Kir6.1 is a transmembrane inwardly rectifying potassium channel regulated by cytosolic ATP. It is responsible for various functions, notably, salt flow and cellular excitability (64). A microinjection of

dominant negative Kir6.1 into developing *Xenopus* embryos will cause a loss-of-function mutation, in turn affecting the morphology of developed tadpoles.

In this study, we will rear *Xenopus* embryos under the aforementioned stressors in conspecific groups and in isolation and compare the frequency of morphogenetic defects in both social conditions.

II. Computational modeling of morphogenetic stress buffering during development

Swarm intelligence and Bio-inspired computing

Mathematicians and computer scientists are tasked daily with solving optimization problems; that is, finding the best possible, feasible solution to a problem at hand. As such problems continue to increase in difficulty, new methods must be explored. Very complex, non-differential, non-converging optimization problems often cannot be solved reliably by traditional methods. There is no better source of inspiration than nature for novel approaches.

Specifically, bio-inspired algorithms often take their inspiration from the swarm intelligence of social animals: a phenomenon in which individual animals take part in social collectives, which maximize productivity through emergent behavior that results from a series of local interactions. Such collectives are able to accomplish feats that individual animals could not accomplish alone. Swarm intelligence (SI) algorithms have been adopted by a wide array of specialties, including reinforcement learning, game theory, and robotics (25, 26). The Bird

Swarm Algorithm is based on social behaviors in flocks of birds, which can be categorized into vigilance, flight, and foraging (27). Glowworm Swarm Optimization was modeled after the collective bioluminescent properties of glowworms, and has shown to be a reliable algorithm for finding multiple optima in multimodal functions (28). The Krill Herd approach takes inspiration from krill foraging behavior based on local interactions between individual krill. It has been applied to various global optimization problems including text mining (29). Many such algorithms are also modeled after eusocial insects, such as ants, (30), bees, (31), and termites (32). All SI-based algorithms share a key aspect: the use of local interactions between agents to create a productive global outcome.

Morphogenesis as emergent behavior

Morphogenesis itself could be seen as a type of collective intelligence; an organism's ability to develop into a target shape occurs due to the interactions of millions of cells, which, individually, would not survive. Moreover, the endogenous pathways underlying various distinct morphogenetic events must interact in such a meticulous manner to accomplish the morphological goal.

Alan Turing, often referred to as the father of modern computer science, was the first to study morphogenesis from a computational perspective; his approach used a reaction-diffusion model (33) . Since this landmark paper, various types of models, including agent-based models, in which many computational "agents" communicate and exhibit emergent behavior, have been applied to morphogenetic systems. Popular categories of such models include proliferation models, differentiation models, and migration models (34).

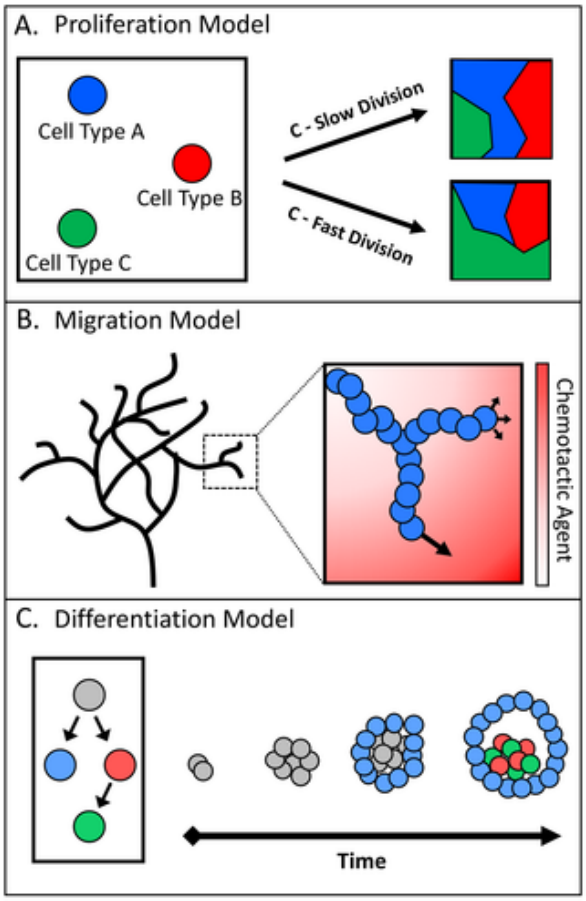


Figure 4. A) Proliferation models focus on the continuous division of classes of cells and the outcome this produces. B) Migration models focus on simulating directed migration of cell types with gradients or other signals. C) Differentiation models show how a progenitor cell type differentiates into various specialized cell types over time, and how those decisions are made. Reproduced from Glen et. al 2019.

Our aforementioned hypothesis (Section I.) supposes that, in the presence of a chemical stressor, there is benefit to developing in a conspecific collective. In other words, there is assistance from neighboring embryos in morphogenesis. According to this hypothesis, morphogenesis is not only emergent behavior in the context of cellular networks, it is also emergent in terms of networks of embryos. Under this theory, while individual embryos undergo internal mechanisms to reach their target morphologies, communication between neighboring embryos

also influences development, resisting the challenge of teratogen. Such behavior provides a fascinating basis for an agent-based model.

Elementary cellular automata

A cellular automaton consists of a grid of cells, each of which can be in one of two states ("on" or "off" for instance). Given an initial configuration, a simple rule dictates the behavior of the automaton: which cells will turn on, and which will turn off? The rules are based on the states of the neighboring cells. Elementary cellular automata follow the same behavior, but in the space of a 1 dimensional array rather than a grid. Different rules yield distinct patterns when run over time. Cellular automata are a fascinating example of emergent behavior in computer science, and can be deemed "agent-based" models in their own right.

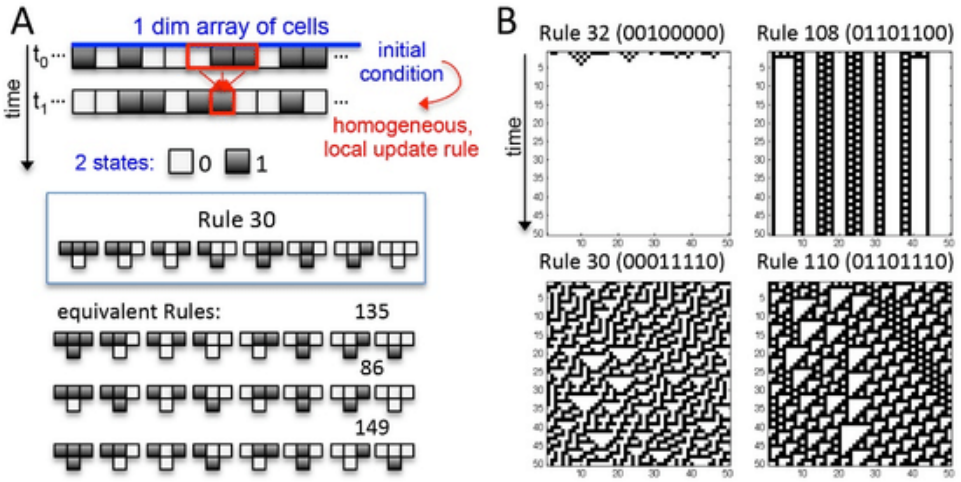


Figure 5. A) Elementary cellular automata are represented by a 1-dimensional array of cells that can either be in one of two states. Over time, they evolve based on a rule. Rule 30 is depicted above; it instructs individual cells to update their states based on the states of their directly neighboring cells. B) Different rule numbers produce different global patterns when run over time. Figure reproduced from Albantakis & Tononi, 2015 (39).

Cellular automata have been widely explored since the 1960s. Since then, they have been applied to games, language and pattern recognition, fault-tolerant computing, geographic information systems, and biology (35). The majority of research in cellular automata theory is focused on investigating the outcome of various rules, categorizing complexity and universality of the results, and applications of their self-organizing behavior (36, 37, 38). While the behavior of a single cellular automaton under various rules and initial configurations has been extensively investigated, there is a lack of literature on the dynamics of coupled cellular automata, let alone networks of them.

Objectives

We propose ECA as a model that mirrors the morphogenetic assistance of *Xenopus laevis* embryo collectives that we hypothesize in Section I. In this model, each embryo is to be represented by a single ECA. We will introduce mathematical noise to simulate the behavior of a teratogen, and will have a network of ECAs running in parallel to reflect a conspecific collective. Communication between simultaneously developing ECAs should help overcome the detrimental effects of the mathematical noise. Over time, the embryo changes configurations according to specified rules, and the final configuration of the embryo is analogous to the final morphology. To simulate the effect of a teratogen, we will introduce mathematical noise by changing the rule, causing the embryo to miscalculate the following configurations. At each time step, there will be a chance for miscalculation: under

the effect of “teratogen,” the chance will be drastically higher. We will model several ECAs, analogous to a collective of embryos, “developing” in parallel, and broadcasting their internal rules to neighboring ECAs at each time step. This communication should prevent high incidence of abnormal “morphology,” even in the presence of “teratogen.” This model encompasses not only individual morphogenesis as a form of emergent behavior at the cellular level with one ECA, but also morphogenesis in conspecific collectives as a form of emergent behavior with a network of ECAs.

Interestingly, the robustness of ECAs to mathematical noise has not been heavily investigated. Therefore, this model will simulate the behavior of a new biological theory while also being impactful to the field of computer science by providing insight into ECA networks, robustness of ECAs to noise, and showing how multi-agent systems respond to noise than individual agents.

III. Stress as a driving force of morphogenesis

Indicators of stress

The word “stress” is a very broad term whose definitions vary tremendously across subdisciplines of biology, let alone fields such as psychology and the social sciences. However, there are a number of agreed-upon ways to quantify stress responses in biology. In organisms with an endocrine system, one method is to measure the concentration of stress hormones in the bloodstream.

The hypothalamic-pituitary-adrenal (HPA) axis is a complex system responsible for synthesis, secretion, and regulation of stress hormones called glucocorticoids. The hypothalamus releases corticotropin-releasing hormone (CRH), which in turn stimulates the pituitary gland to release adrenocorticotropic hormone (ACTH), which targets the adrenal cortex and triggers the release of glucocorticoids (40, 41). In amphibians, the glucocorticoid levels can be quantified from a tissue, blood, or excretions (42).

Alternatively, many biomarkers of stress at the cellular level exist. Thermal stress, oxidative stress, heme release following injury, dehydration, apoptosis, DNA damage, and misfolded proteins are all conditions that trigger the expression of various stress-related biomarkers (43, 44).

The link between stress and development

Stress hormones and other biomarkers have shown to be vital to normal development. CRH has proven to be crucial during vertebrate life stage transitions that involve significant morphological changes, including the hatching of turtles and chicken, smoltification of salmon, and anuran metamorphosis.

CRH is upregulated during such life stage transitions. Moreover, inhibition of endogenous CRH in bullfrogs resulted in a delay of morphological changes during metamorphosis such as front limb development, body size increase, and tail height. (45). In humans and rats, glucocorticoids are essential for the maturation of the kidneys, liver, lungs, and nervous system in *utero* (46, 47, 48, 49).

Heat shock proteins (HSPs), which are induced after a traumatic event such as injury or thermal stress (50), have been shown to have a role in many morphogenetic transitions. The RNA transcript for *Hsp70* was up-regulated throughout limb regeneration in axolotls (51). Moreover, Mexican blind cavefish, which do not have visible eyes due to extreme dark habitats, appear to have HSP90-dependent eye morphology changes. When river fish embryos were moved to a simulated dark, cave-like environment, *Hsp90* was upregulated, and there was a significant increase in both eye size and eye socket size variation in adult river fish raised in the cave-like environment compared to adult river fish raised in river-like environments. These data suggest that physiological stress is a capacitor for morphological evolution of eye size (52). In fruit flies, it has been shown that reduced HSP90 results in a heritable altered chromatin state that leads to abnormal eye phenotypes, indicating an epigenetic mechanism by which stress is a catalyst for morphological changes (53). Additionally, homozygous *Hsp90*ß-mutant mouse embryos developed normally until stage 9-9.5, after which they died due to failed trophoblast differentiation needed for the transition from the yolk sac to the placental labyrinth (54).

Protein disulfide isomerases (PDIs) are involved in mediating apoptosis due to misfolded proteins (55). In mice, heterozygous *Pdia3* deletion resulted in embryonic lethality. Homozygous Pdia3 deficient mice showed impaired bone formation due to dysfunctional osteoblast differentiation (56). In a mouse model, inhibition of PDIA3 resulted in failed terminal differentiation in myogenesis during muscle regeneration (57).

Heme-oxygenase 1 (HMOX1) is an enzyme that degrades heme and is a marker of oxidative stress. It is critical for erythroid development during embryogenesis (58). It is involved in apoptotic prevention and cellular proliferation, linking it to the progression of many different cancers (59).

Remarkably, stress markers appear to cycle throughout normal development in Xenopus laevis, and spike during certain transitions. Namely, PDIA3, HMOX1, and HSP90 exhibit cyclic increase over early embryonic development (61).

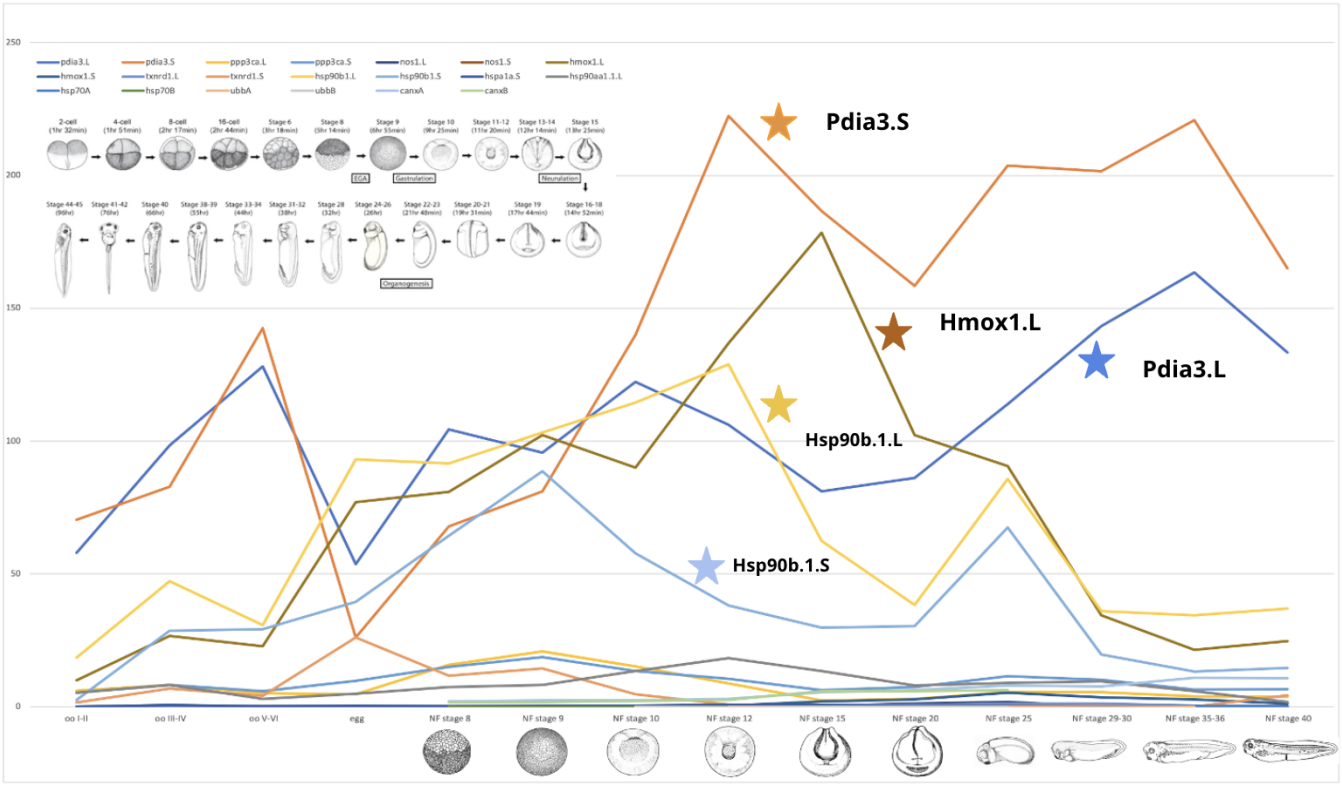


Figure 6. A study of gene expression during embryonic development showed that *Pdia3*, *Hmox1,* and *Hsp90* genes, all biomarkers of stress, go through peaks and valleys of expression at different morphological transitions. Figure courtesy of Angela Tung.

Questions and objectives

Existing literature strongly suggests that stress biomarkers are upregulated during morphological transformations, and associated proteins have a notable role in mediating these transitions. This begs the question: at each stage in development, is it “stressful” to stray from your target morphology? Does a stress response indicate that your current morphological state is not where it should be, triggering the next stage of development? To explore this theory, we propose comparison of stress marker profiles between embryos with induced abnormal morphologies and their age-matched embryos.

Xenobots, synthetic living proto-organisms which are created by surgically removing the animal cap of a *Xenopus laevis* embryo at NF stage 7-9.5 (60), are an example of self-organizing a novel morphology. The “stress” of extraction and losing expected positional information signals is an interesting concept to be explored. Moreover, as these proto-organisms form their novel morphologies, become ciliated, and swim around, does this result in decreased stress levels, as the “target morphology” has presumably changed?

An common example of morphogenetic abnormality that occurs during early embryonic development is exogastrulation. Gastrulation is the process in early development that results in the formation of the three germ layers: ectoderm, mesoderm, and endoderm. Exogastrulation occurs when the endoderm, which should develop inside of the embryo, moves outward. This is a deviation from the target morphology. According to our theory, exogastrulation should result in higher stress levels.

Finally, injury during development is a traumatic event that should trigger stress biomarker expression. Over the course of remodeling and regeneration, is this stress maintained due to being morphologically challenged? *Hsp70* expression over the course of axolotl limb regeneration suggests that this is possible (51). To test this theory, stress marker quantification during tail regeneration in tadpoles can be analyzed.

METHODS AND MATERIALS

Embryo collection and maintenance

Healthy embryos were selected from clutches of fertilized eggs at the 2-4 cell stages and placed in dishes of 0.1x MMR. They were kept in a 14˚C incubator and monitored daily. Media was changed every other day. The size of the dish in which embryos were kept was dependent on the size of the group.

Thioridazine treatment

Thioridazine was obtained from Sigma Aldrich and dissolved to a 90 µM concentration. Thioridazine was added to the media when embryos were at NF stages 12.5-13. 40 mL of the drug was added to the media for every 100 embryos in the dish. Embryos were subsequently removed from the drugged media and placed back into 0.1x MMR at around NF stage 25.

Microinjections of DomNeg Kir6.1

Bubble pressure between 55-60kPA was used and injection pressure of 140kPa. A chimeric construct dominant negative for Kir6.1 was microinjected at the 2-cell stage. Stock was diluted at 2:5 in nuclease free water. Injection was at the 100 ms setting.

Scoring of morphological abnormalities

Morphological defects were visible at NF stage 45 under a microscope. Tadpoles were placed in tricaine to prevent movement during observation. Defects were observed qualitatively and a record was kept. To prevent bias, “blind” scoring was done, in which the scorer was unaware which group they were scoring.

Animal cap extraction

Fertilized embryos which were kept at 14˚C in 0.1x MMR were taken between NF stages 7-9. The embryos were transferred to a 1% agarose-lined petri dish with 0.75x MMR media. Surgical forceps were used to remove the vitelline membrane of the embryos, followed by a small, thin piece of the animal cap. These animal cap explant were placed in a fresh agarose dish with 0.75x MMR. The remaining portions of the embryos were discarded. Animal caps were kept at room temperature for one hour and allowed to heal into a spherical mass of cells. They were then kept in the 14˚C incubator. The animal caps were transferred to new media daily to clean out lingering debris.

Tail surgery

Tailbuds (NF stage 35-37) were exposed to tricaine as an anesthetic. A surgical scalpel was used to make a straight cut removing the last millimeter of the tail. Tailbuds were then moved back into tricaine-free 0.1x MMR media, placed in the 14°C incubator, and allowed to regenerate for 7 days.

Exogastrulation

Embryos were placed in high-salt (1x MMR) media until the end of gastrulation.

RNA extraction, cDNA sythesis, and qPCR

RNA extractions were performed using Qiagen kits, and cDNA synthesis was performed with BioRad kits.

Imaging

All images were taken on a Nikon camera scope using the Micro Manager software.

Computational Modeling

Models were written in Python using Jupyter Notebooks(65). Parts of the code were inspired by Vladimir Ilievski's Medium article on elementary cellular automata (66). Rule 184 was used as the state transition function for elementary cellular automata for its powerful ability to converge to a repeating pattern.

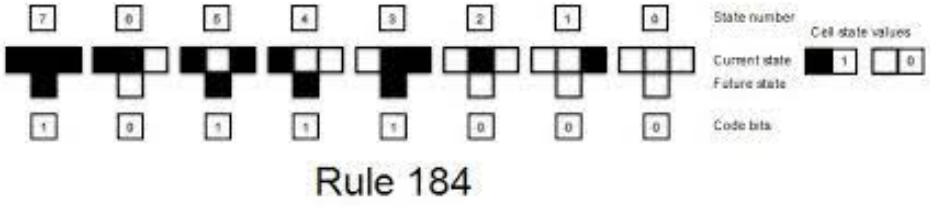


Figure 7. Rule 184, encoded by the binary number 10111000, instructs state transitions of each cell based on the states of immediate neighbors. Figure reproduced from Clemson University Department of Mathematics website.

To represent mathematical noise, analogous to the effects of a teratogen, we had a function that altered Rule 184 by flipping a bit at random based on a predetermined probability of interference. Teratogen would be analogous to a high probability of interference, whereas untreated controls would be analogous to a very low probability of interference. This essentially means that ECAs representing teratogen-treated embryos are very likely to have their rules altered, but ECAs representing untreated embryos are unlikely to have their rules altered, allowing them to reach the expected configuration.

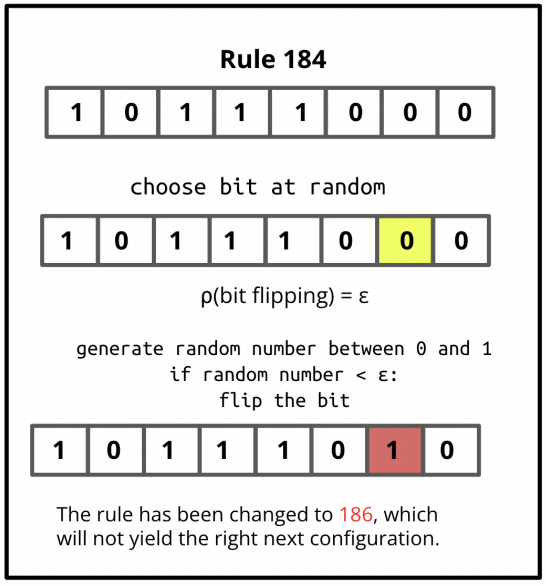
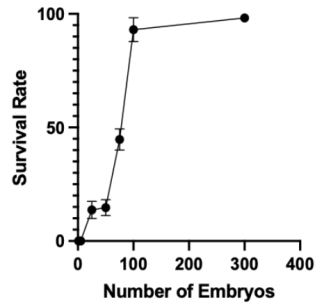


Figure 8. Pseudocode for introducing mathematical noise in ECAs.

RESULTS

I. Developing in a group offers a higher chance of survival against thioridazine exposure

A



B

Group Size	Corrected Concentration (uM)
n=25 media	48.816
n=50 media	50.764
n=100 media	43.876
n=200 media	46.6256
n=300 media	48.312
n=400 media	45.46

Figure 9. A) Plates of 1, 5, 25, 50, 75, 100, and 300 embryos, respectively, were treated with thioridazine at NF stage 12.5-13. There is a significant increase ($p < 0.05$, Paired T-test) in survival rate from n=50 to n=75 and another significant increase from 75 to 100. **B)** There is no significant difference ($p > 0.05$) between the corrected concentrations of the drug across different sized groups, confirming that this effect is not due to larger groups metabolizing the drug faster.

Embryos were reared in groups of variable sizes from 1-300 embryos per petri dish. Embryos from different groups in the same experiment replicate were all from the same females. Thioridazine was added to the media at NF stage 12.5-13, and embryos were subsequently removed from the drug and placed back into 0.1x MMR at stage 25. The percent survival of the embryos after the treatment was observed and recorded. Embryos drugged alone or in groups of 5 showed no survival. Groups of 25 and 50 showed a very low survival rate with no significant difference between the two groups. There was a significant increase in survival rate between the 50-group and the 75-group, as the group of 75 had an average survival rate of 46.667%. Another drastic increase was seen between the 75-group and the

100-group, as the 100-group had an average survival rate of 93%. There was no significant difference between the survival rates of the 100-group and the 300-group. These data suggest that larger groups confer protection against thioridazine-induced lethality (Fig. 9).

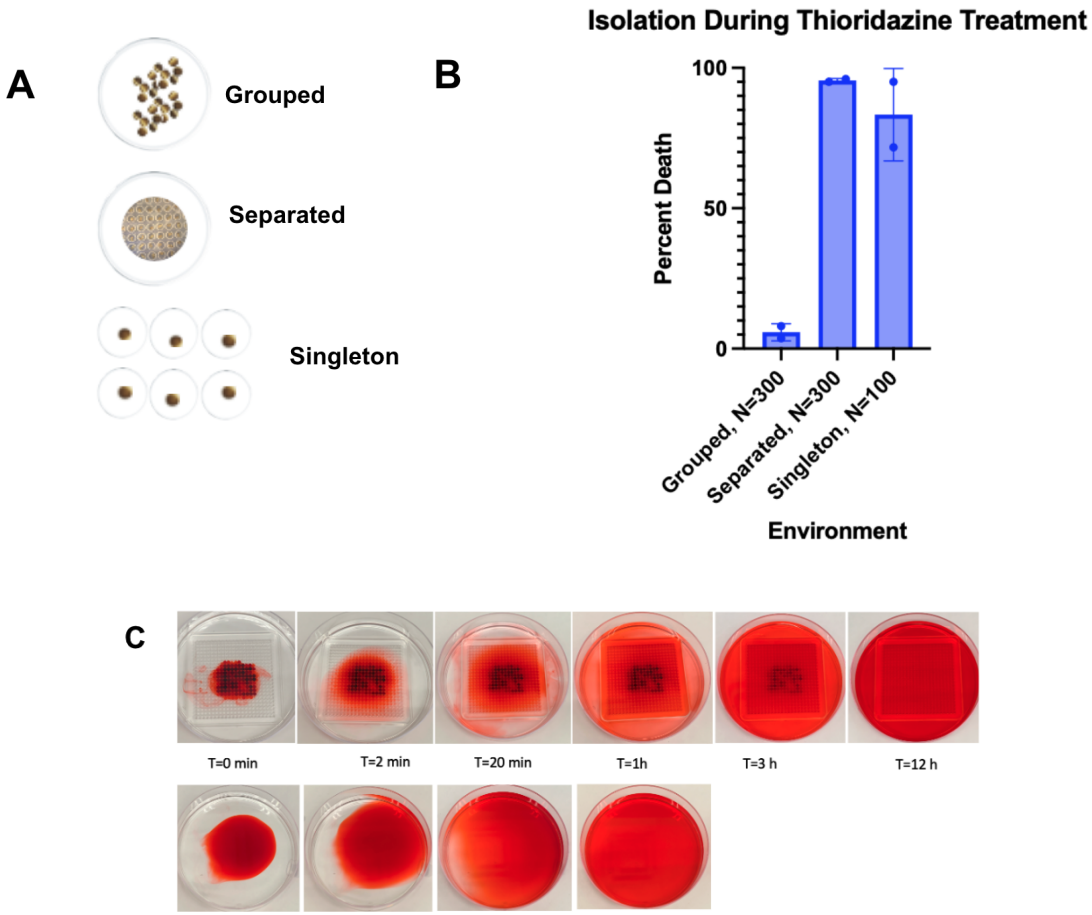
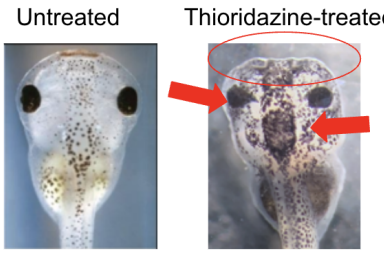


Figure 10. A) Embryos were either reared in a plate together with no barriers (grouped), reared in the same plate with a physical barrier that slows diffusion of compounds in MMR (separated), or placed alone, one per well in a 6-well dish (singleton). **B)** Separated and Singleton treatments resulted in major lethality, while there was high survival in Grouped treatment. There was no statistically significant difference between Separated and Singleton. C) The acrylic barrier used for the Separated group allowed diffusion of compounds through the MMR media, but diffusion was significantly slowed.

During the treatment phase (from thioridazine exposure at NF 12.5-13 through removal from the drug) embryos were either kept in a group, physically

separated in shared media, or completely isolated with different media (Fig 10A). Grouped embryos were placed in the same plate in shared media with no barriers. Separated embryos were placed in the same petri dish and media, but were physically separated by a 3D-printed acrylic barrier, in which each embryo was confined to a 1mm radius well. The barrier allowed for diffusible molecules to pass through the shared media, but diffusion occurred more slowly than without the apparatus (Fig 10C). Singleton embryos were placed one-per-well in a 6-well dish. Both kinds of isolation resulted in drastic death rates following thioridazine treatment, while embryos that were freely grouped together showed a very low death rate. These data suggest that separation during teratogen exposure is lethal.

II. Larger group sizes exhibit lower frequency of the "square-headed" defect caused by thioridazine exposure



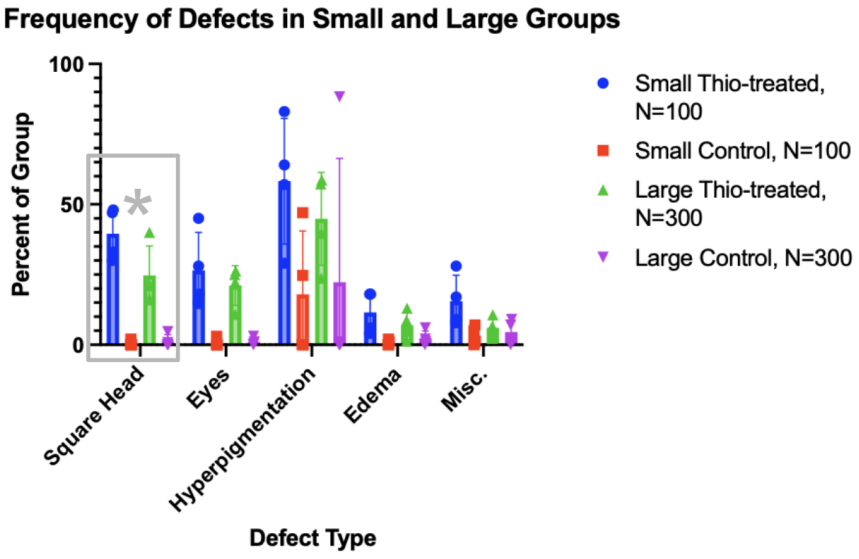


Figure 11. Thioridazine treatment caused many morphological aberrations, including a square head shape, a "raindrop" eye morphology, hyperpigmentation, edemas, and other defects. For all defect categories, there was a statistically significant ($p < 0.05$, Student's T-test) between treated groups and untreated groups. Notably, there was a statistically significant difference between the N=100 thioridazine-treated group and the N=300 thioridazine-treated group for the square head defect, in which the square-head defect had a lower incidence in the larger group.

Thioridazine treatment caused a wide array of observable morphological defects in tadpoles. Remarkably, a morphogenetic rescue effect was observed with the square head phenotype: groups of 300 embryos had a lower incidence of the

square head-morphology than groups of 100 embryos. This suggests that larger group sizes may confer resistance against the formation of this particular defect. However, it should be noted that this rescue effect was not observed with significance with any of the other defect categories (Fig. 11).

III. Conspecific morphogenetic rescue effect observed during DomNeg Kir6.1 Microinjection

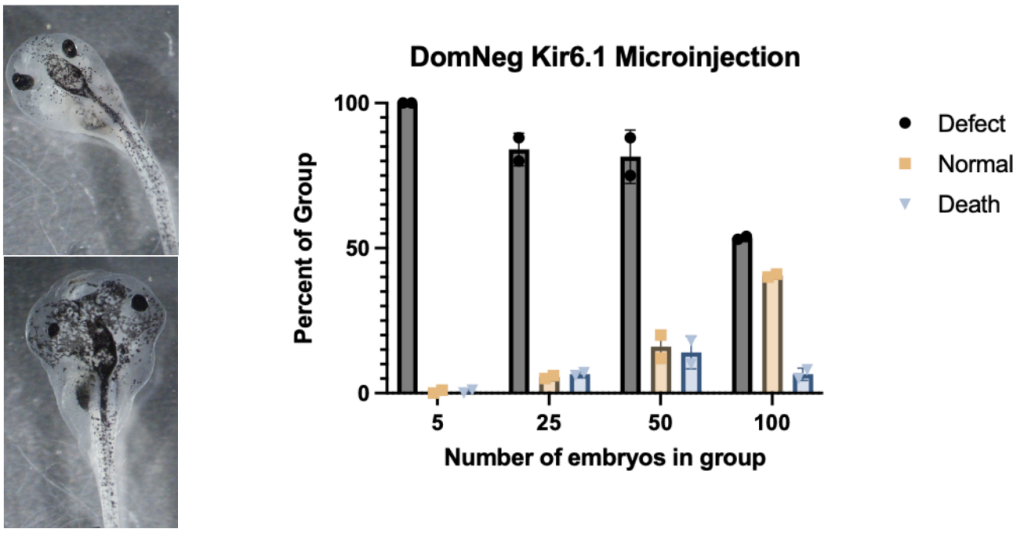


Figure 12. Microinjection of dominant negative Kir6.1 causes noticeable morphological defects in tadpoles (left). Injected embryos were either in groups of 5, 25, 50, or 100. A very low rate of death was observed among all groups. A significantly lower percentage of the 25-group and 50-group were defected in comparison to the 5-group ($p < 0.05$, Student's T test). The 100-group had a drastically lower defect percentage than all other groups.

Embryos microinjected with dominant negative Kir6.1 often developed into tadpoles with morphological abnormalities, such as craniofacial geometry, deviation from symmetry, eye shape and hyperpigmentation. The two

medium-sized groups showed significantly lower defect rate than the smallest group, however, they still had very high defect rates. The largest group showed significantly lower defect rates than all smaller-sized groups (Fig. 12), suggesting that higher presence of conspecifics does help while developing with induced mutations.

IV. Introduction of untreated individuals into the population modifies the morphology of both treated and untreated groups

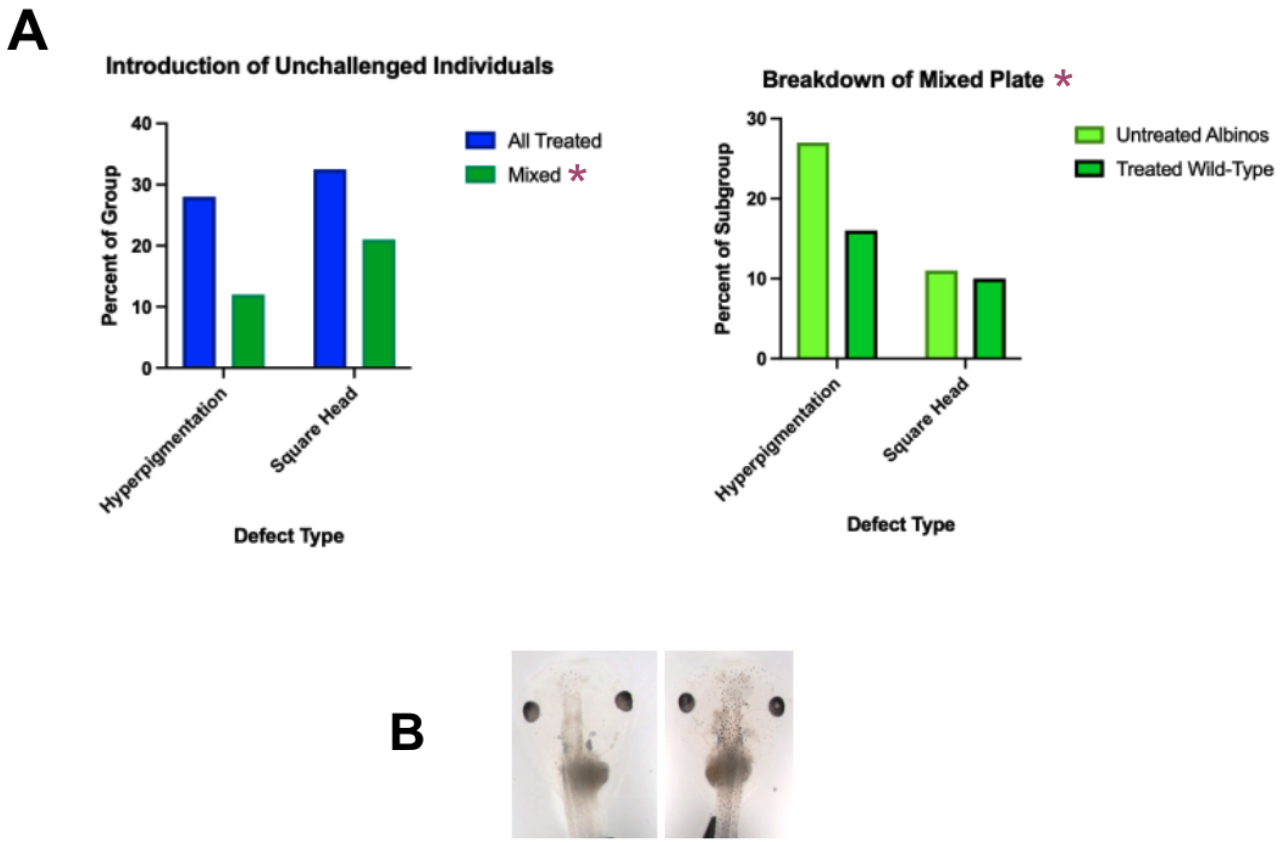


Figure 13. A) Untreated embryos were introduced into a plate of treated embryos. Albino embryos were used as a marker for untreated embryos. In the mixed plates, treated embryos showed a lower-than-usual incidence of defect, while untreated albinos showed high incidence of defect. Percentages in the right-hand graph were calculated like such: (# defected albinos / total albinos * 100) **B)** Hyperpigmented albinos were observed in the mixed plates: no pigment cells are observed on normal albino tadpoles (left), however, some tadpoles in mixed plates showed small clusters of pigment cells (right).

To further investigate the effect of conspecific cues on morphogenesis, we introduced untreated individuals into a plate of embryos that had been treated with thioridazine. Albino embryos were used as the untreated subgroup for easy visual classification of treated vs untreated embryos. Plates of 100 embryos that were treated with thioridazine were reared in parallel to mixed plates of 200 untreated albinos and 100 treated wild type embryos.

The mixed plates had lower incidence of defects than the non-mixed plates, which was mathematically expected, however, the breakdown of the mixed plates showed a few fascinating phenomena. We observed a fairly high frequency of hyperpigmented albinos and square-headed albinos in the mixed plate, while treated embryos showed lower incidence of defects than the fully-treated plate. This suggests a two-way street of conspecific cues, in which defective embryos detrimentally affect unchallenged embryos, and unchallenged embryos positively affect defective embryos.

V. Computational modeling

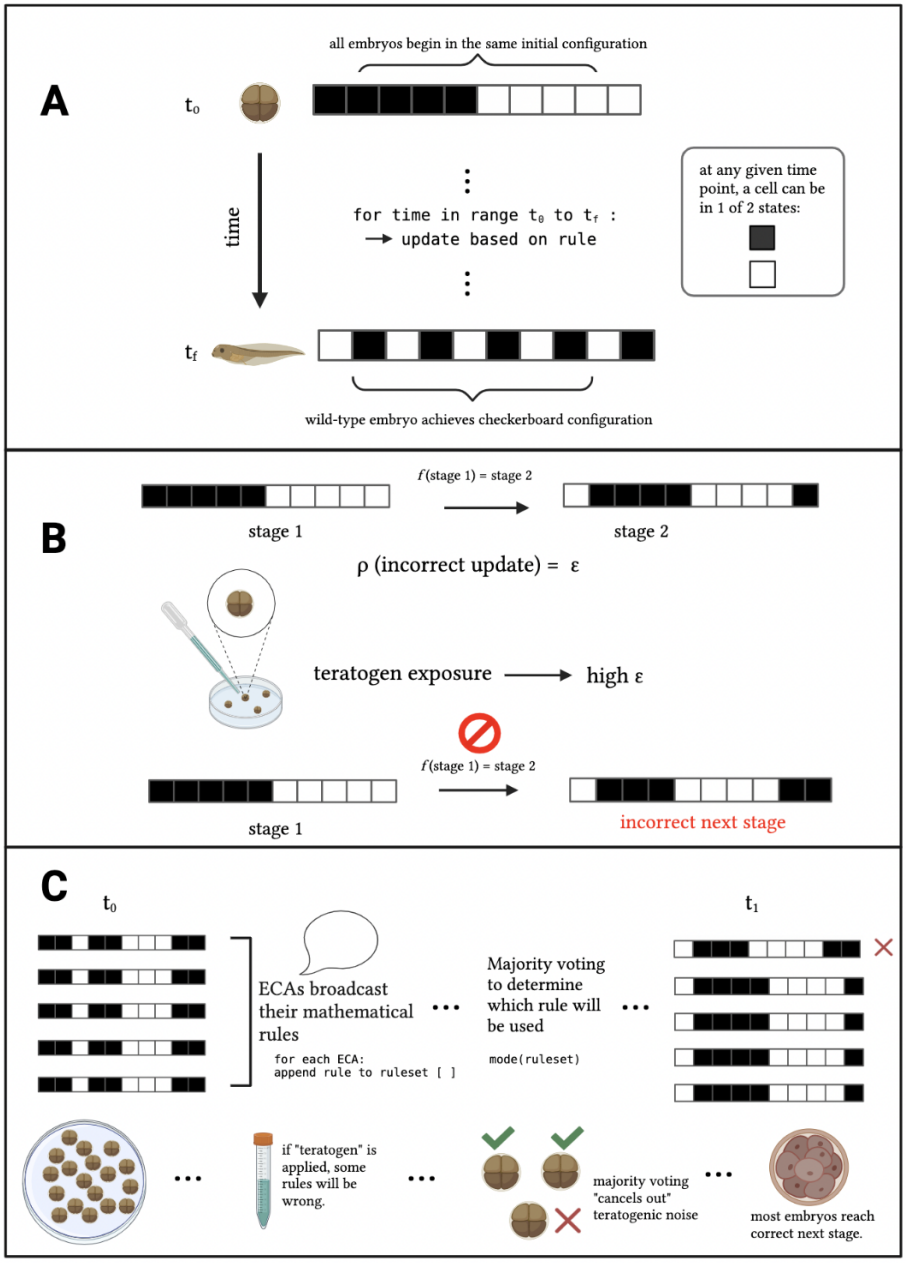


Figure 14. A) An initial configuration of an ECA is analogous to an early embryo. An initial configuration must have 50:50 ratio of on and off states. For a fixed number of time steps, the majority problem will be solved, analogous to wild-type embryonic development. **B)** At each time step, a mathematical function involving bitwise manipulation will allow evolution to the next stage. There will always be a probability of an incorrect update. For wild-types, this probability will remain low, but for teratogen-exposed embryos, the probability will be high, most likely causing incorrect evolution. **C)** A group of embryos is represented by a network of ECAs that all begin in the same initial configuration. At each time step, they exchange their rules; that is, the most common rule in the set of rules will be used for evolution. This will "cancel out" high noise and result in most of the embryos correctly progressing to the next stage of development.

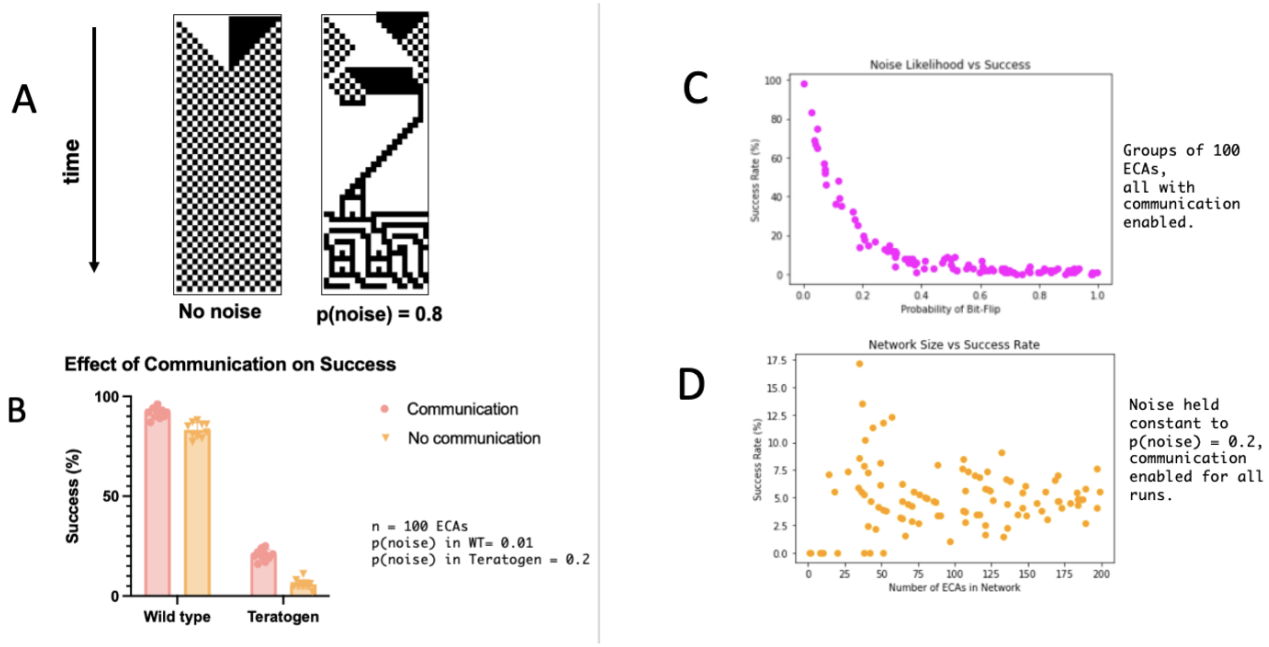


Figure 15. A) ECA using Rule 184 converges to a checkerboard pattern (left) over time. Mathematical noise introduction prevents this convergence (right). **B)** Networks of 100 ECAs with low noise (wild-type) and high noise (teratogen) are observed with and without enabling communication. Allowing information exchange significantly increases the success rate in the teratogen group ($p < 0.05$, Student's T-test). **C)** In communication-enabled networks of 100 ECAs, there is a strong inverse relationship between strength of noise and rate of success. **D)** There is no strong correlation between an increase in network size and success rate after 50 ECAs.

We created a model of an ECA network in which baseline behavior is defined by Rule 184, but introduction of mathematical noise alters the rule, hindering desired transitions. After a parameter search, we determined that a probability of rule alteration of 0.2 best reflects teratogen treatment, and that of 0.01 best represents a lack of treatment. Addition of communication within an ECA network allows ECAs in the network to exchange information about which internal rule they are using, perform a "majority vote" to determine the best rule, and correct their behavior accordingly (Fig. 14).

Rule 184, with our initial configuration, should converge to a “checkerboard” pattern. We measured the success of each ECA by whether its final configuration was a “checkerboard,” or alternating “on” and “off” states, or not. ECAs with high noise probability fail to meet this success criterion. We showed that there is a significant difference in success rate between “wild-type” networks and “teratogen” networks, and that introducing communication leads to a statistically significant increase in the success rate of “teratogen-exposed” networks. Moreover, we showed the strong inverse relationship between the strength of the noise and the success rate in groups of 100 ECAs with communication ability. This confirms that our simulation of teratogen works as desired. However, we did not find a strong correlation between the success rate of a communicative network and the number of ECAs in the network- we did not observe a steady increase in success as we increased the size of the network as we expected to.

VI. Stress marker analysis

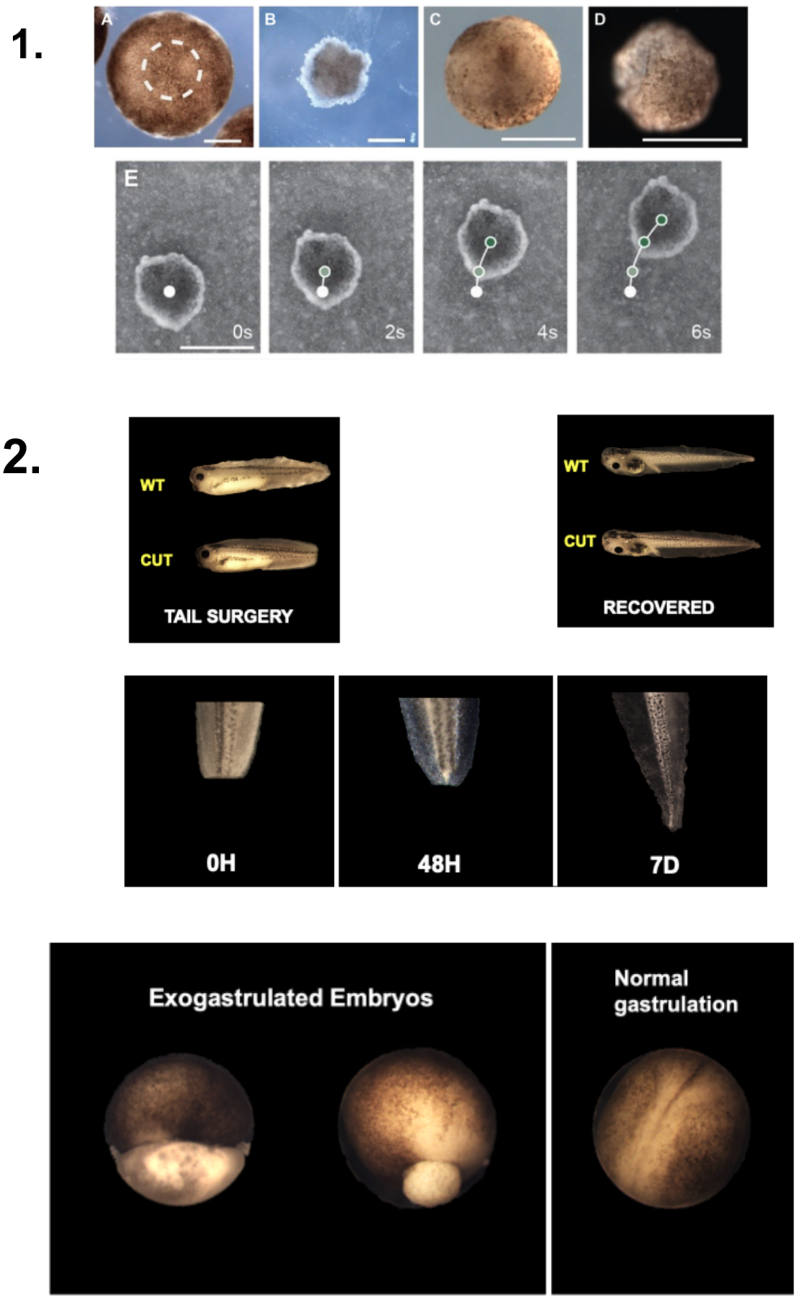


Figure 16. 1) A piece of the animal cap is extracted from the early embryo (A), allowed to heal (B), and forms a spherical mass (C) . After 4 days, it becomes ciliated (D) and exhibits movement (E). Panel 1 reproduced from Blackiston et. al 2021. **2)** Removal of a tail fragment around stage 33 leads to a 7-Day regeneration period in which the tail grows back in full and normal development continues. **3)** Exogastrulation occurs when embryos fail to internalize their endoderm and is visible through a microscope.

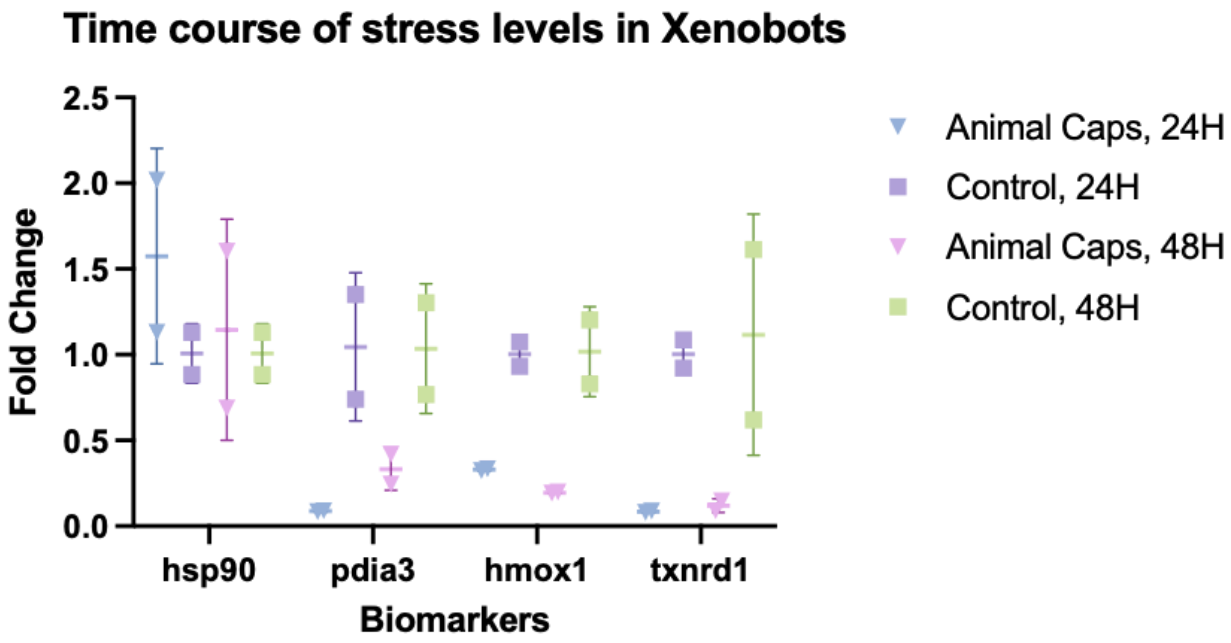


Figure 17. Stress marker analysis from qPCR data for 24 hour animal caps and 48h animal caps compared with the controls. Hsp90 is upregulated in animal caps at both time points, and other stress markers are downregulated.

Animal cap removal, tail surgery, and exogastrulation were carried out and documented (Figure 16). While COVID-related supply shortages in cDNA synthesis kits caused a delay in our analysis, our preliminary results show that Hsp90 is upregulated in removed animal caps at 24 hours and 48 hours post-surgery. Fascinatingly, the other 4 stress markers, Pdia3, Hmox1, and Txnrd1 are downregulated in extracted animal caps (Figure 17).

DISCUSSION AND CONCLUSION

In this paper, we studied external influences and endogenous stress signals involved in morphogenesis using *Xenopus laevis* embryo model. Noting the many examples in nature in which morphological changes ensue based on social circumstances, and the stress-buffering effect exhibited by conspecific collectives, we approached morphogenesis through a lens of emergent behavior.

We sought to investigate effects of developmental challenges on embryos reared in a collectives of different sizes versus in isolation. We hypothesized that, in line with the stress-buffering effect, embryos reared collectives would be more likely to overcome developmental obstacles. To test this hypothesis, we exposed groups of embryos to the teratogen thioridazine, known to cause craniofacial defects. Remarkably, we noted that separation during treatment was lethal, and that being in a group conferred significant protection against thioridazine exposure. As the number of embryos in a group size increased, the survival rate increased, with a near-total survival rate observed around 100 embryos in a collective. We also observed a "rescue" effect: large groups (300 embryos) had a lower incidence of the thioridazine-induced square-head defect than small groups (100 embryos). However, it should be noted that this rescue effect was not seen with the other defect categories with significance. Additionally, we microinjected embryos with Dominant Negative Kir6.1, inducing a loss-of-function mutation in their inwardly rectifying potassium channels, which in turn causes morphological defects. We observed a fascinating effect where, as the number of embryos in a recovering group increased, the percentage of defects greatly decreased.

To explore these phenomena further, we hope to identify the communication signals between developing embryos; it could be communicated optically, through physical touch, or through a diffusible molecule. Moreover, we seek to identify when, during the course of development, the signals are exchanged.

We subsequently explored the effect of introducing unchallenged embryos into plates with challenged groups to see whether untreated embryos would provide morphogenetic assistance. We used albino embryos to visually identify which embryos in mixed plates were untreated. Fascinatingly, while we did see lower defect incidence in the mixed plates, we noticed a “two-way street” effect: untreated albinos were showing fairly high rates of hyperpigmentation and square-headedness. This leads us to infer that, while unchallenged individuals may help challenged individuals, the reverse effect may also take place. It will be necessary to tease apart these signals. It may also be necessary to observe how albinos and wild-type embryos interact at a baseline, in the absence of any teratogen, to get a better understanding of the albino hyperpigmentation effect.

Collective intelligence in nature has inspired many computational algorithms which seek to solve complex optimization problems. Our investigations suggest emergent behavior that maximizes resistance to stressors; this lends itself well to a computational approach. We developed an ECA-network model of the observed behavior. We used mathematical noise to simulate the effect of teratogen and communication between ECAs as a means of overcoming this noise. Our model reflected the observations that show communication between individually developing agents significantly increases the success rate of the whole network in

the face of stress. However, it does not appear that success rate increases steadily with the number of ECAs in the network. In future developments, we will modify our computational representations of communication until we see a global result that reflects our experimental findings.

Pandemic-related shortages in supplies needed for caused a delay in our data collection regarding differential stress marker expression, thus, we were unable to perform analysis in time for the Senior Honors Thesis deadline. However, we have been able to obtain preliminary results. Interestingly, we see a downregulation of stress in all markers except Hsp90 from our preliminary stress marker analysis. It should be noted that HSPs, Hsp90 in particular, have been linked with morphogenesis more often than other stress markers. We predict that stress levels will decrease throughout the time course, and expect to see a change in stress marker expression at our 5-day time point, as this is when morphology changes drastically with mucociliation. We note that Xenobots are unique in that it is possible their target morphology changes after removal from the embryo, as their final form is a mucociliated proto-organism. Furthermore, we predict that, with the 7-day time series of tail regeneration, we will see an increase in stress post-surgery, and a gradual decrease as animals grow closer to their target tails. We predict that stress will be upregulated in exogastrulated embryos in comparison to normal gastrulation. We have collected all samples necessary for this analysis and await delivery of materials necessary for expression analysis.

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