A LITERATURE REVIEW OF UNISEXUAL *AMBYSTOMA* AND RELATED POLYPLOID HYBRID SURVIVAL AND DEVELOPMENT

by

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for eight credits.

To my best friends, the most blindly supportive group of humanities majors I know.

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ABSTRACT

The *Ambystoma laterale-jeffersonianum* complex is a collection of salamander populations inhabiting a significant portion of New England, including western Massachusetts. This complex consists of two groups, sexuals and unisexuals. Sexual *A. laterale* and *A. jeffersonianum* are diploid and reproduce sexually. Unisexuals, conversely, range from haploid to pentaploid, and are simply classified as *Ambystoma*, having no true species identity. This population of unisexual salamanders consists entirely of females which can reproduce both sexually and asexually (Bogart et al, 2007). Within this complex, unisexuals can sexually reproduce only with sexual males, often resulting in hybrid offspring (Charney et al., 2014). Due to the incongruence of ploidy between sexuals and unisexuals, hybrid offspring range in ploidy from haploid to pentaploid as well. While hybrids can develop into completely healthy, phenotypically normal adults, they face an extremely high mortality rate as embryos prior to the first cleavage event (Avis, 2019; Charney et al., 2019).

Throughout my research, I have amassed information regarding the *Ambystoma* complex's embryogenesis, genetics, and environment in an attempt to consolidate much of what is currently known about them in the literature. In addition, I here propose multiple experiments that could be completed by future thesis students and other researchers in order to determine what factors play a role in causing this high mortality rate in early hybrid embryos.

INTRODUCTION

The *Ambystoma laterale-jeffersonianum* complex of New England features a combination of sexual and unisexual salamanders, whose unisexual population relies on the sexual population for both sexual and asexual reproduction. Through sexual reproduction, unisexual and sexual gene pools interact, generating hybrid offspring ranging in ploidy from haploid to pentaploid. Though these hybrids can mature into viable, phenotypically normal adults, the vast majority die during early embryogenesis, around the time of the first cleavage event. The cause of this mortality is currently unknown. Here, I will discuss embryonic development of different animals, possible genetic or environmental explanations for *Ambystoma* hybrid embryo mortality, and steps that may be taken to investigate this phenomenon in the future.

Figure 1. **Four *Ambystoma* species male salamanders**
From left to right, these salamanders are *A. jeffersonianum*, *A. tigrinum*, *A. laterale*, and *A. texanum.* (Bogart et al., 2007).

SECTION 1: DEVELOPMENT

General Vertebrate Development

Having an understanding of vertebrate development is equally as crucial to having a fundamental understanding of biology as it is exciting. Its branches reach into every area of the life sciences, being heavily influenced by cellular and molecular biology, endocrinology, genetics, and evolution, among many others. Vertebrate development is a foundational pillar of biology that is evidenced by a myriad of observable organisms in nature, as well as ourselves. This has been a discipline of great focus for thousands of years, with this being of interest to the Babylonians, Egyptians, and Greeks for example, being studied by everyone from Aristotle to Leonardo da Vinci (Horder, 2010).

On a larger scale, the study of vertebrate development and embryology has been pertinent to the fundamental curiosities of humanity, attempting to answer questions of how life is created and how we come to be. Developmental biology paints a picture of both our understanding of the natural world, but also of ourselves, throughout history. The progression of human understanding of embryonic development has been dramatically accelerated through the use of rapidly-advancing technology. It was about 1677 when Antoni van Leeuwenhoek

first viewed sperm cells following his own invention of the microscope, when the theory that gametes exist as preformed homunculi reigned supreme (Horder, 2010). Now, less than 350 years later, we have gained a significant understanding of the mechanisms that govern development on a cellular and molecular scale, and possess the technology to visualize developing organisms in great detail and even to manipulate their genetics.

Using our knowledge of embryonic development, in addition to that of other related fields of biology and environmental sciences, we can attempt to answer many questions posed to us by the natural world, such as our questions of *Ambystoma* hybrid embryo mortality.

General Embryonic Development

In discussing embryonic development, there are several points at which it becomes difficult to generalize. For example, many species of fish and amphibians use external fertilization in order to reproduce, whereas terrestrial mammals use internal fertilization. Additionally, in cases of internal fertilization, the generation of a zygote may occur relatively instantaneously, or sperm can be held in the body for extensive periods of time prior to a fertilization event (Gilbert, 2010). There is also a large degree of variability regarding periods of embryonic development; in the case of *Drosophila melanogaster*, development from fertilization until hatching takes between two and four days, whereas the

African elephant has a gestation period of almost two years (Gilbert, 2010). Additionally, there is significant variability between the development organs and specific body parts across different animals. For example, humans' neural development process during embryogenesis is significantly prolonged due to our disproportionately large, complex brains, while a tardigrade's brain only contains 200 neurons (Martin et al., 2017). Despite this large breadth of differences, however, I will attempt to summarize embryonic development in the most universal terms.

Generally, embryonic development begins with fertilization, in which a sperm cell enters an egg cell, forming a zygote. Gametes can be united through a series of different mating practices, and in most mammals, this occurs internally, whereas in most non-mammals, it occurs externally (Gilbert, 2010). After fertilization, several cleavage events occur, increasing the amount of cells in the embryo exponentially. As the cells begin to grow in number, the embryo becomes a morula, a stage at which a solid sphere of cells is formed (Gilbert, 2010). These cleavage events signify the blastulation stage, in which embryonic cells begin to differentiate. The outer layer of the mass of cells becomes the trophoblast and the innermost cells become the inner cell mass, or the embryoblast. The embryoblast then forms an embryonic bilaminar disc. Its upper layer is made of epiblast, or primitive ectoderm, and its lower layer is made of hypoblast, or primitive endoderm (Gilbert, 2010).

By this point in development, many organisms have completed the process of the maternal-to-zygotic transition. This is a process in which the developing embryo transitions from translating mRNAs and using proteins provided by the mother to transcribing mRNAs from its own genome, thereby synthesizing its own proteins. This new state following the maternal-to-zygotic transition is referred to as embryonic genome activation (Tadros & Lipshitz, 2009).

Next, the primitive streak is formed on the embryonic disc by cells from the ectoderm, signifying the beginning of gastrulation. As this streak continues to expand, the primitive groove appears on its surface. The sides of the primitive streak give rise to the mesoderm, the germ layer existing between the endoderm and the ectoderm (Gilbert, 2010). Typically, the ectoderm gives rise to the epidermis, as well as hair, nails, skin, and hooves in animals that possess them. The mesoderm gives rise to several internal structures including smooth muscle, connective tissue, blood vessels, reproductive organs, and endocrine glands. The endoderm gives rise to elements of the respiratory and digestive systems (Gilbert, 2010).

In addition to the formation of the three germ layers, several other events may happen during gastrulation as well, depending on the animal. One such event is epiboly, where the outermost layer of ectodermal cells moves and grows to surround the mesoderm (Gilbert, 2010). Another is invagination, a process which forms the blastopore during this stage in development through an infolding of

cells into the center of the embryo. If the developing embryo is a deuterostome, this invagination will go on to become the anus, and if it is a protostome, it will go on to become the mouth (Gilbert, 2010). In pseudopods, this occurs as a migration of individual cells into the embryo (Gilbert, 2010). During gastrulation, cells across the embryo begin to differentiate and express different genes, thereby losing their totipotentiality (Gilbert, 2010).

Following the formation of the primitive streak, neural folds develop behind the embryonic disc and extend backward. These two folds join together, and between them develops the neural groove. Over time, this groove becomes deeper, with the neural folds growing upward. In vertebrates, this becomes the process of neurulation, where the neural folds then grow together and form the neural tube, the first evidence of an embryo's nervous system (Gilbert, 2010). After this has occurred in vertebrates, the opening of the blastopore now leads to the neural tube, establishing the neurenteric canal and enabling communication between the yolk sac and the amniotic sac (Gilbert, 2010). Next, the neural folds come together around the area of the hind brain, coalescing until they close at the anterior end of the brain. Before the neural groove closes entirely, a group of ectoderm cells, called neural crest cells, accumulate at the neural folds. These cells will go on to develop the ganglia of the head, spine, and sympathetic nervous system. It is also around this time that two tubes formed by the mesoderm converge in front of the buccopharyngeal region of the embryo. As these tubes

come together and twist, they create the heart and its four chambers in mammals and birds, three chambers as in amphibians, or two as in fish (Gilbert, 2010).

In addition to neurulation, other forms of organogenesis also take place following the differentiation of germ layers. However, from this point forward, the organogenesis of different animals does not follow a distinguished pattern. Instead, different animals may develop different organs at different intervals, in different orders. From the processes of organogenesis and morphogenesis, most animals develop into larvae. Once these larvae hatch, they later undergo the process of metamorphosis, in which juvenile structures break down and adult structures are developed (Gilbert, 2010). In animals which do not develop into larvae, live birth often occurs. Here, the majority of structures present at birth are maintained into adulthood and are generally not broken down.

Amphibian Embryogenesis and Development

The majority of amphibian eggs are fertilized externally, meaning that a male will fertilize eggs which have already been laid in an aquatic environment, by depositing sperm directly onto them. These eggs, like many others, are polar prior to fertilization, meaning they have a dense yolk at their vegetal pole and little yolk located at their animal pole (Gilbert, 2010). Embryonic development begins at the opposite side of the egg from where the sperm enters. This is the site where the dorsal lip of the blastopore will form, and where the gray crescent

begins to form and wrap around the embryo about one hour following fertilization (Hill, 2020). The gray crescent is the location at which gastrulation begins. It also provides insight into the future body plan of the amphibian; the animal pole above the crescent will form the anterior, the vegetal pole below it will form the posterior, the widest part of the crescent will form the dorsal region, and the narrowest part of the crescent will form the ventral region (Gilbert, 2010). This is not present in all amphibians, such as the model organism *Xenopus*, but those that lack the gray crescent will develop similarly. The location where the sperm enters the egg will give rise to the ventral part of the embryo, with the opposite side being the location where the dorsal and spinal part of the embryo will be formed (Gilbert, 2010).

Though *Xenopus* does not exhibit the typical gray crescent in its embryonic development, it can be used in a laboratory setting to study embryogenesis due to its ability to live in captivity, large embryo size, and its capacity to mate several times within a year, among many other qualities (Garvey, 2000). Due to the large size of their eggs and embryos, they are an ideal model for cellular division, as development can be easily visualized. The first cell division that occurs during embryonic development does so about three and a half hours following fertilization, in the north-south direction down the animal pole, and the second division occurs in the same manner, with the second division beginning before the first finishes (Hill, 2020). The third cleavage event occurs equatorially,

though it occurs around the animal pole rather than being at the true equator of the embryo. This unequal division occurs because the animal pole contains less yolk, meaning that dividing here is physically easier to accomplish due to its lack of density (Gilbert, 2010).

As subsequent cleavages occur, the animal pole continues to experience more divisions than the vegetal pole for this reason. Eventually, the meridional divisions will span the entire embryo, but as these divisions occur several equatorial divisions also take place, primarily at the animal pole (Gilbert, 2010). Through embryonic development, the vegetal pole will not catch up to the amount of divisions that the animal pole undergoes, leaving fewer cells. This low number of divisions at the vegetal pole also explains why cells at the animal pole are significantly smaller than those at the vegetal pole during blastocoel formation (Gilbert, 2010).

When the embryo contains between 16 and 64 cells, it is in the morula stage, occurring directly before the blastula stage, where the blastocoel, a cavity filled with fluid, forms in the embryo. This occurs when the embryo has reached a total of 128 cells, which is after about seven divisions (Gilbert, 2010). The cells of the embryo are held together by embryonic cadherins, which become increasingly important as the cells begin to grow in number. When cadherins are removed, the appropriate structures do not form, and instead, the embryo will fall apart (Gilbert, 2010).

After about 12 divisions, at the mid-blastula transition stage, the embryo undergoes the maternal-to-zygotic transition, triggered by changes in chromatin. From here, several more changes occur, ultimately leading to gastrulation (Gilbert, 2010). *Xenopus* has historically been used as a model organism to study these processes. Similarly to other organisms, gastrulation occurs as a means to transport cells inside the embryo which are destined to form the endoderm and subsequently the internal organs, to envelope the embryo in ectodermal cells, and to generate a mesoderm which exists as a layer between the two (Gilbert, 2010). Because of the polarity of the initial egg cell, these germ layers may be mapped and tracked even prior to the fertilization event. The ectoderm originates from the surface of the animal pole, the endoderm originates from the surface of the vegetal pole, and the mesoderm originates from the internal cytoplasm (Gilbert, 2010).

Gastrulation occurs at about 26 hours after fertilization in the area of the gray crescent, below the equator (Hill, 2020). The embryo then undergoes an invagination, forming the blastopore. Cells lining the opening of the blastopore and the developing archenteron undergo a morphological change, becoming bottle cells. Bottle cells are the first cells to significantly change shape during embryogenesis (Gilbert, 2010). In *Xenopus*, gastrulation begins at the marginal zone, at the equator of the blastula. While the first cells to make up the dorsal lip of the blastopore are bottle cells, this changes rapidly as cells continue to travel

into the embryo. As more cells travel inward, the blastocoel is moved to the side of the embryo which is opposite of the blastopore lip. At this time, the blastopore lip expands dramatically, a ventral lip forms, and the only exposed endoderm exists as the yolk plug, which is then internalized. Now, the ectoderm covers the entire surface of the cell and the endoderm exists internally, with the mesoderm being located between the two (Gilbert, 2010).

Next, the process of neurulation begins with the neural plate being formed at the mid-dorsal region of the embryo. The neural folds form from the ectoderm along the neural plate, growing in size and coalescing to form the neural tube. The neural tube then closes, later going on to form the spinal cord and brain (Gilbert, 2010). The cells connecting the epidermis to the neural tube then detach and become neural crest cells, which later form melanocytes, neurons of the peripheral nervous system, and cartilage structures within the face. Following the formation of the neural tube, organogenesis continues. Mesodermal tissue running along the notochord differentiates into somites, which will go on to form the dermis, spinal cord, and back muscles. The embryo then develops its mouth and the body begins to elongate, developing into the recognizable tadpole structure. Next, neurons make connections between each other and to muscles throughout the body. Soon following is the formation of the gills, signifying the tadpole's readiness to hatch, which it will do at about 140 hours following fertilization, once it depletes the supply of yolk provided by the mother (Gilbert, 2010).

Following hatching, several subsequent developmental processes must occur in order for the tadpole to develop into a mature *Xenopus* adult. This entails the degeneration of gills, the growth of embryonic teeth, the formation of internal gills, and a refinement of preexisting organs. After this, the hindlimbs begin to develop, as do the forelimbs shortly after. Next, the tail is absorbed and reduced, and following, metamorphosis is complete. After this process finishes, the adult emerges from the water as a frog (Gilbert, 2010).

Comparison with Mammalian Development

Historically, studying mammalian embryonic development has been challenging, as a mammalian egg cell, such as a human egg cell, is less than one one thousandth of the size of a *Xenopus* egg (Gilbert, 2010). One mammalian model organism which has been more heavily experimentally studied is the house mouse, *Mus musculus*, which has a primordial oocyte diameter which is one third of the size of a human's, also making it extremely difficult to study (Hill, 2019).

As with other mammals, mice reproduce via sexual reproduction, in which an egg is fertilized internally. After sperm enters the female mouse, it approaches the egg cell which has been released from the ovary, with the speed of their flagella beating increasing as they pass through the corona radiata, the outermost layer of the egg cell. They then make contact with the zona pellucida, a membrane beneath the corona radiata, triggering the acrosome reaction and allowing the

sperm to fuse with the secondary plasma membrane of the oocyte. This results in two blocks to polyspermy, ultimately allowing only one sperm cell to enter into the egg (Hill, 2019). This initially occurs as a depolarization of the oocyte, and then later as a wave of calcium from the sperm cell fused with the secondary plasma membrane. This causes cortical granules from within the oocyte to come to the surface of the secondary plasma membrane and release their contents, triggering a chemical reaction that ultimately renders the zona pellucida impermeable to other sperm cells. In the case of the house mouse, their point of fertilization is marked as occurring 12 hours after its initial release of the oocyte from the ovary (Hill, 2019).

As the sperm cell travels into the oocyte, the oocyte undergoes meiosis II, developing into a female pronucleus. At the same time, the sperm cell develops into a male pronucleus. Upon their fusion, the zygote is formed. At this point, the egg cell has entered the ampulla and is surrounded by sperm cells. After fertilization occurs, two nuclei form within the zygote about one day post-fertilization (Hill, 2019). At 36 hours, all sperm cells have fallen away from the zygote, and the unicellular zygote has divided into two cells. At two days, the two cells have both divided, creating four cells. At two and a half days, these cells have all divided again, creating eight cells. At three days, these cells compact and tight junctions form between them: this creates the morula (Hill, 2019).

Between the third and fourth days, the embryo enters its hatching blastocyst phase, where the zona pellucida falls away, and the inner cell mass continues to compact within the trophoblast, or the outermost layer of the blastocyst. As these two distinct regions of the embryo form, cells continue to divide. After the fourth day, the embryo enters the late blastocyst stage, where the inner cell mass becomes the epiblast, which is pushed toward the outer surface of the embryo. This will later differentiate into the mesoderm. At this stage, the hypoblast also differentiates; this will go on to form the endoderm. At four and a half days, the embryo finishes its journey down the ampulla and implants in the uterus (Hill, 2019).

On the fifth day, the embryo takes on the egg cylinder morphology and the ectoplacental cone appears. At this point, the embryo sees a rapid growth in the amount of cells within the inner cell mass, which leads to the epiblast formation. At five and a half days, the anterior-posterior axis is determined through nodal signaling, which causes the formation of the distal visceral endoderm. On day six, the advanced endometrial reaction occurs, and at six and a half days, heart precursor cells begin to localize at the midline of the epiblast (Hill, 2019). At one week, the amniotic fold forms, and at seven and a half days, the amniotic cavity distinctly forms three cavities: the exocoelomic cleft, the ectoplacental cleft, and the amniotic cavity. At this point, the neural plate becomes defined at the anterior end of the embryo. Then, the allantoic bud begins to grow considerably and the

neural groove becomes visible. At this time, head fold growth continues, and foregut pocket formation begins (Hill, 2019).

On the eighth day, between 14 and 16 somites form, with the neural tube closing around the fourth and fifth somites. At this time, the first branchial arch begins to form, which will later give rise to the pharynx. Additionally, the otic pit begins to drastically develop, becoming more indented into the head; this will give rise to the internal structures of the ear (Hill, 2019). At about eight and a half days, the heart tube from the mesoderm begins to twist around itself, creating the structures of the heart, and the anterior neuropore forms and closes. At the ninth day, the posterior neuropore forms and the forelimb buds begin to become apparent. Next, the posterior neuropore closes; this will go on to form the forebrain. Additionally, the hind limb bud and the tail bud become visible, and the nasal cavities begin to form (Hill, 2019).

On the tenth day, the lens pit begins to develop and deepen; this will give rise to the lens of the eye. At the same time, the umbilical hernia forms due to the rapid development of the midgut, and the tail begins to lengthen and thin (Hill, 2019). Otic ganglion cells also begin to form, and differential gene expression begins to occur in the gastrointestinal tract. Additionally, the heart continues to develop, constructing the outflow tract system, and development of the renal system begins (Hill, 2019). On the eleventh day, the lens vesicle closes, the nasal pits begin to form, and nerve endings within the ears begin to develop. At the

same time, gonads begin to differentiate and blood flow becomes supported by the right ventricle of the heart. At 11 and a half days, the lens vesicle separates from the ectoderm and becomes entirely closed, and other parts of the eye become developed (Hill, 2019). The forelimbs become continually longer and more defined, with the limb-girdle and handplate structures being visible, although individual digits are not yet differentiated. The otic pit also becomes more defined and decreases in size. Epithelial cells begin to migrate into the gonads, and in biologically male mice, these will become Sertoli and interstitial cells of the testes. During this time, the placode of the teeth, the genital systems, the mammary glands, and the chambers of the heart begin development (Hill, 2019).

At 12 days, the digits of the paws begin to form, and the handplates and footplates begin to assume an angular shape. At this point, the corneas, brain vesicles, and tongue are all formed and visible. Gonads also physically differentiate between biological females and males, and oocytes in the ovaries of biologically female mouse embryos begin to enter the first meiotic prophase (Hill, 2019). There is also the formation of cartilage at some joints throughout the body. Additionally, teeth enter the bud stage, and the septums of the heart continue to develop. At 13 days, the elbows and wrists become defined. The pinna of the ears grow rapidly and the vibrissae, or whiskers, become visible on the face. During this time, the liver also begins to develop, the cells of the gastrointestinal tract

experience differential gene expression, and the heart continues to develop (Hill, 2019).

Beginning at 14 days, the digits of the front paws separate and the limb bones are developed. Additionally, hair follicles become present across the body and the umbilical hernia becomes less dramatic. At this point, the thyroid is formed, the developing teeth enter the cap stage, and lymph node development begins (Hill, 2019). At 15 days, the digits of the feet separate and become visible, the teeth enter the bell stage, and mammary tissue continues to develop. At sixteen days, nails form on digits, eyelids develop, and the umbilical hernia begins to disappear. Additionally, the valves of the heart are modified and the liver continues to develop. At 17 days, the skin thickens and the umbilical hernia disappears completely. Epithelial cells also begin to develop into prostate buds in biologically male mice. At 18 days, whiskers lengthen and become thicker and hair cells begin to differentiate. At day 19, the mouse is born (Hill, 2019).

After birth, several developmental changes must occur before the mouse is fully matured. Hair does not begin to grow on the body until several days after birth. Similarly, the ears and eyes do not open until between one and two weeks after birth (Hill, 2019).

Comparison with *Drosophila melanogaster* development

Much of what we know regarding how genetic and molecular processes function originates from research done on the fruit fly *Drosophila melanogaster*. *Drosophila* development significantly differs from that of mammals and amphibians. While fertilization also occurs internally, this is followed by an oviposition event where up to 500 eggs are laid in or near a food source (Loppin et al., 2015). A single sperm enters the egg, and following the formation of the male pronucleus, the male and female pronuclei fuse to generate a diploid nucleus. Male pronucleus formation is triggered by the genes *Hira*, *yemanuclein*, and *chd1*. Almost immediately, this single-celled zygote's nucleus undergoes several rapid divisions, thereby developing into a syncytium, where several nuclei are present in a single cell (Loppin et al., 2015). After eight cleavages, the 256 nuclei migrate to the outer edges of the cell, forming a cellular blastoderm. During this migration, they experience two more divisions, creating a total of 1,024 nuclei, with the majority of these nuclei entering the cell's cortex. Differing drastically from the development of larger organisms, this all occurs in about the first 90 minutes post-fertilization (Gilbert, 2010).

Still existing as a syncytium, the cortical nuclei are able to express the genes transcribed by RNA Polymerase II, allowing them to synthesize proteins. At the same time, the cortical nuclei divide three more times, creating a new total of about 6,000 nuclei within four hours of the initial fertilization event (Gilbert,

2010). As the cells grow in number, the time between their divisions increases: while the first ten divisions each only take about eight minutes, the next three divisions take about 25 minutes. The fourteenth division is asynchronous, with the time of this division taking between 75 and 175 minutes; it is around this time that the embryo experiences embryonic genome activation, meaning that transcription of genetic material within the nuclei is greatly increased (Gilbert, 2010)

As this occurs, proteins in the early embryo begin to form concentration gradients which dictate how further stages of development will progress. For example, bicoid and hunchback proteins are found at high concentrations at the anterior end of the embryo while caudal and nanos proteins are found at high concentrations at the posterior end of the embryo (Gilbert, 2010). At the same time, other genes called gap genes dictate the segmentation which will occur later within the life of the fruit fly. These include *knirps*, *giant*, *tailless*, *Krüppel*, and *huckebein*, and are expressed at different longitudes throughout the body (Gilbert, 2010). The expression of these genes within the embryo is heavily dictated by the maternal mRNAs and proteins provided during oogenesis. Mutations or absences of these genes may result in extreme phenotypic deviations from wild type *Drosophila*, historically being mapped onto the genomes of other species as a means to study genetic mutation in larger organisms (Gilbert, 2010).

During this time, gastrulation begins, initiating the differentiation of the ectodermal, mesodermal, and endodermal germ layers. The population of about

1,000 cells that will go on to compose the mesoderm make up the midline across the ventral side of the embryo. This midline then folds inward, forming the ventral furrow, which eventually forms the ventral tube within the embryo, later flattening out to form the mesoderm. Around this time, the embryo also curves slightly, forming the cephalic furrow. As development continues, the germ band is formed through ectodermal and mesodermal cells migrating to the ventral midline; following the development of the germ band, cells designated for specific fates are now located appropriately within the embryo. Additionally, the embryo now divides into body segments, separating sections of ectoderm and mesoderm. After this, segmentation, organogenesis, and separation and development of imaginal discs occur. The nervous system also begins to develop from the ectoderm on the ventral side of the embryo; in insects like *Drosophila*, the nervous system is localized to the ventral region of the embryo, differentiating themselves from vertebrates, whose nervous systems are derived from a neural tube that forms at the dorsal region of the embryo (Gilbert, 2010).

During this time, the body segments become distinguished, forming a head, three thoracic segments, and eight abdominal segments. While legs eventually grow from all three thoracic segments, wings will only grow from the mesothorax. Ultimately, when the *Drosophila* larva hatches from the egg, it maintains an incredibly simple body plan. A great deal of development into the

adult fruit fly does not take place until later, during metamorphosis from the larval stage into the final adult stage, similarly to amphibians. (Gilbert, 2010).

Salamander Development

One example of a salamander outside of the *Ambystoma* genus is the four-toed salamander *Hemidactylium scutatum*, another species native to eastern North America. These salamanders' eggs are fertilized internally, and following this fertilization, multiple females often deposit their eggs into a single nest, where one female tends to them (Alford et al., 2013). In total, *H. scutatum* undergoes 28 stages of embryonic development, differing from *A. mexicanum*'s 40 embryonic development stages and *A. laterale* and *A. jeffersonianum*'s 27 (Schreckenberg, 1975). The first 19 stages of *H. scutatum* embryonic development follows first cleavage through neurulation, and stages 20 through 28 involve developments of appearance and external morphological changes (Hurney et al., 2015).

Prior to fertilization, the egg cell is heavily polarized, with the germinal vesicle being located at the animal pole. About eight hours following fertilization, the first cleavage event begins at the animal pole and the furrow extends meridionally, creating two cells. As this first cleavage event is occurring, a second cleavage furrow begins to form perpendicular to the first furrow, extending through the animal pole. Following these divisions, four blastomeres are formed (Hurney et al., 2015).

At 18 hours, a third cleavage furrow forms, dividing the four cells into eight equatorially. This division, however, does not occur directly along the equator and instead occurs toward the animal pole, creating an asymmetry such that the cells at the animal pole are significantly smaller than those at the vegetal pole. After this, cell divisions continue, creating a total of 16 blastomeres, forming a morula (Hurney et a., 2015). Asymmetrical cell division continues, creating 32 cells at around 24 hours, with the cells at the animal pole being about half the size of animal pole cells at the 16-cell stage. Cell division then continues further, creating 64 cells at 27 hours. The cells at the animal pole become about half the size of those at the 32-cell stage (Hurney et al., 2015).

At one day and 16 hours, the early blastula forms, creating a hollow ball of cells. At the same time, cell division is asynchronous and continues to generate increasingly small animal blastomeres. As cells continue to divide, the embryo enters its late blastula stage, where the number of cells becomes effectively impossible to quantify (Hurney et al., 2015). Following the blastula stage, it enters the early gastrula stage at three days and six hours, evidenced by the formation of the dorsal lip of the blastopore. This blastopore lip then elongates, later shortening to create a deep slit within the embryo at five days (Hurney et al., 2015).

At seven days, the dorsal lip of the blastopore elongates. The neural plate becomes elevated from the surface of the embryo, and a neural groove forms at its anterior end. At seven days and six hours, the neural groove deepens and the

neural folds become visible. At the same time, the dorsal lip of the blastopore decreases in size (Hurney et al., 2015). Next, the neural folds continue to emerge and grow toward each other, and the neural groove is fully formed at about eight days. At eight days and three hours, the neural folds begin to fuse near the boundary between the midbrain and the hindbrain (Hurney et al., 2015).

Next, the regions of the developing brain become visible and the neural folds continue to coalesce at nine days. At nine days and three hours, the neural folds have become fused along the length of the embryo and more development of neural areas begins to occur (Hurney et al., 2015). The head fold begins to become visible at this point. At ten days, the suture of the neural tube becomes smooth and neural development progresses, with the anterior brain regions enlarging. The head fold becomes more prominent, distinguishing the head from the rest of the body (Hurney et al., 2015). At 11 days, the gill mound emerges from the branchial plate. At this point in time, the blastopore exists as a slit in front of the tail bud, which begins to emerge from the body. At the same time, optic vesicles become visible on either side of the neural tube. At about 14 days, the gill mound and forelimb bud continue to grow in size, and the head becomes undercut, forming gill arches. At this point, the heart is formed and the embryonic heartbeat is visible (Hurney et al., 2015).

Starting at 18 days, the gill stalk divides into three stalks, pigmentation of the optic cup develops, and the nasal pits become formed. Additionally, the heart

continues to develop, and the tail continues to lengthen. At 22 days, the gillstalks become elongated and melanophores become distributed throughout the dorsal surface of the body. At the same time, the optic cup continues to develop, and the growing structures of the jaw fuse together. At 25 days, melanophores begin to increase in concentration in the head, torso, and tail regions, forming pigmented spots which will later be visible in the larva. The hindlimb bud also becomes visible and grows in size during this stage. At 28 days, gills become branched and patterns of pigmentation are visible and continue to become more distinguished as the concentration of melanophores increases. It is also around this time that the elements of the forelimb become differentiated, developing wrists and elbow joints. During this stage, the hindlimb bud forms a paddle-like appendage. At 33 days, forelimbs form three distinct digits. Pigmentation through the body increases, leading to a dark stripe forming through nasal pits and eyes laterally along the head. The gut becomes recognizable at this time, forming an s-curve shape. At about 37 days, the forelimbs now have four digits and the elbow joints are fully functional. Finally, at 45 days, gill slits elongate, the nervous system finishes formation, and the gut continues to become more complex in shape (Hurney et al., 2015). The mature adult morphology comes about later after a period of metamorphosis.

Ambystoma Development

Because of their threatened status and abnormal methods of reproduction, the development of salamanders of the *Ambystoma laterale-jeffersonianum* complex has not been studied in detail in comparison to other organisms such as *Xenopus*. However, their unique reproductive practices and earliest stages of development have been covered in the literature.

Even before the event of fertilization, *Ambystoma* development marks itself as unique from that of other amphibians for two major reasons. First, unisexual *Ambystoma* females are kleptogens, meaning that the spermatophores they utilize for sexual reproduction are deposited into vernal pools for the use of *A. laterale* or *A. jeffersonianum* females to use in sexual reproduction (Charney et al., 2019). Second, while the majority of amphibians reproduce sexually, there are three methods through which *Ambystoma* unisexual salamanders may reproduce: normal sexual reproduction, gynogenesis, and hybridogenesis, with gynogenesis being the most common. Gynogenesis is a process in which the female *Ambystoma* unisexual takes up sperm for the purpose of activating embryonic development, although the paternal DNA is not incorporated into the offspring (Charney et al., 2019). In hybridogenesis, conversely, eggs are fertilized by paternal sperm, although this genetic material is not passed on to subsequent generations (Charney et al., 2019). In each of these methods of reproduction, the

female unisexual takes up spermatophores which have been deposited in the water of a vernal pool via the cloacal lips (Charney et al., 2014).

In events of sexual reproduction and gynogenesis, the unisexual *Ambystoma* female are thought to undergo pre-mitotic endomitosis. In these instances, the G1 phase of the cell cycle is excluded, causing a doubling of chromosomes within the egg cell without a cellular division. For example, this can allow for an initially haploid egg to become diploid or even tetraploid prior to fertilization (Charney, et al., 2019). Haploid eggs being combined with haploid sperm will create a diploid zygote, whereas diploid eggs combining with haploid sperm will form a triploid zygote and tetraploid eggs combining with haploid sperm will generate a pentaploid zygote.

After mating, *A. jeffersonianum* females typically lay eggs in one to two days (Kipp, 2000). In the case of the unisexual *Ambystoma* salamanders, however, a female can store eggs internally for an indefinite amount of time (Charney et al., 2014). One possibility proposed as an explanation for hybrid embryo mortality suggests that eggs become overripe before they are laid, and it is possible that spermatophores stored within the female might be impacted by a similar degradation (Charney et al., 2019).

Once the female decides to lay eggs, it will create egg masses, which typically feature between 20 and 30 eggs in the case of *A. jeffersonianum*, although they can contain anywhere between one and 60 (Kipp, 2000). In natural

conditions, eggs may take up to 14 weeks to hatch, although higher temperatures can accelerate this process, meaning that eggs laid later in the breeding season will give rise to salamanders much more rapidly than those laid at the beginning of the breeding season (Kipp, 2000).

Additionally, another unique phenomenon of early *Ambystoma* hybrid embryonic development pertains to embryo mortality. During embryonic development, hybrid embryos see a dramatic rate of mortality prior to the first cleavage event, although the exact rate is not certain. Diploid *A. laterale* and *A. jeffersonianum* embryos, however, do not see this high mortality rate, and following the first cleavage event, the rates of survival of all embryos regardless of ploidy and genomotype remains low and uniform (Charney et al., 2019). Exactly what causes this high rate of mortality in early *Ambystoma* hybrid embryos is currently unknown. In the following sections of this thesis, I will suggest many possible genetic and environmental factors which may play a role in this hybrid embryo mortality.

SECTION 2: GENETIC FACTORS

Genetic Analysis of *Ambystoma* Salamanders

Over the course of embryonic development, many genes and proteins play a role in the transformation of the zygote to a larva. One such gene is *vegt*, a transcription factor which codes for VegT, a protein that controls fate maps of the presumptive endoderm in early amphibian embryos, including those within the *Ambystoma* genus. The *vegt* gene comes in two forms, maternal *vegt* (*mvegt*) and zygotic (*zvegt*), which are expressed in high levels during early embryogenesis (Sudou et al., 2016). *mvegt* mRNAs can be found in the vegetal hemisphere of amphibian embryos at the time of first cleavage, the time in embryonic development related to my study. Because *mvegt* is provided by the mother whereas *zvegt* is encoded within the genome of the embryo, I will focus on *mvegt*, as I am concerned with the effects of VegT prior to the first cleavage event, which occurs prior to embryonic genome activation, before *zvegt* transcription begins (Sodou et al., 2016).

The *vegt* gene and the subsequent VegT protein that it encodes are significant within the process of embryonic *Ambystoma* development because of

its role in developing the future gut, as well as its hypothesized role in dorsal development. It has been shown that following fertilization, VegT is conserved to the vegetal pole in healthy *Xenopus* embryos at the earliest stages of development, as well as other amphibian species, likely influencing the development of the dorsal region of the body (Sudou et al., 2016).

In an instance where mutations have occurred in *mvegt*, or where translation of *mvegt* mRNAs occurs improperly, it is possible that the fate maps of developing embryos could become heavily distorted during early stages of development, possibly leading to embryonic mortality as seen in *Ambystoma* complex hybrid embryos. This is especially true considering the high polarity of amphibian embryos, shown by the high concentration of yolk at the vegetal pole and minimal yolk at the animal pole (Gilbert, 2010). If VegT is distributed more evenly throughout, it is more highly concentrated at the animal pole, this may lead to a heavily distorted fate map and may trigger apoptosis, ultimately resulting in embryonic mortality.

Another genetic factor is the EP-cadherin protein, a cell adhesion molecule in early amphibian embryos. In the case of *Xenopus*, this protein exists in the embryo as both a maternal mRNA and a protein product prior to fertilization, and exists to hold cells together in the embryo as it develops (Ginsberg et al., 1991). In the event that an excess of the EP-cadherin is synthesized early on in development, embryos are left unable to properly divide,

and if this occurs later in development, morphogenesis may be seriously impacted, resulting in embryonic deformation (Gilbert, 2010). Alternatively, a lack of EP-cadherin may result in obliteration, in which the cells of the embryo become detached from one another, killing the organism (Gilbert, 2010).

In the event that ploidy is correlated with mortality, meaning that *Ambystoma* hybrids with higher ploidy counts have a higher embryonic mortality rate than those with lower ploidy counts, EP-cadherin could be a key factor. Because of the abnormally high amount of genetic material being transcribed and translated within each cell of polyploid hybrid embryos, it is possible that an insufficient amount of EP-cadherin is translated because the amount of protein-synthesizing machinery is not adequate for the amount of mRNAs that are available to be translated at a given time.

Because there is a significant portion of the *A. laterale* and *A. jeffersonianum* genome which is still unknown, it is impossible to amass a definitive list of all genes or proteins which may contribute to *Ambystoma* hybrid embryo mortality. Ultimately, this signals a necessity to sequence the genomes of both species, as well as to study the genomic makeup of *Ambystoma* hybrid embryos.

Maternal-to-Zygotic Transition

The maternal-to-zygotic transition is an event in which the *Ambystoma* embryo shifts from translating mRNAs and using proteins implanted into the egg by the mother to transcribing mRNAs from its own genetic material. Through the maternal-to-zygotic transition, the zygote enters zygotic genome activation, also called embryonic genome activation, where maternal mRNAs and proteins are eliminated (Tadros & Lipshitz, 2009). In *Ambystoma*, this occurs early on in the development of the zygote, during the mid-blastula transition, and is crucial for survival into the larval stage (Charney et al., 2019). It is also hypothesized that early embryogenesis, and the event of zygotic genome activation more specifically, faces a risk of being compromised within *Ambystoma* hybrids embryos. These embryos are especially susceptible to abnormalities in development, apoptosis, and mortality in general (Charney et al., 2019).

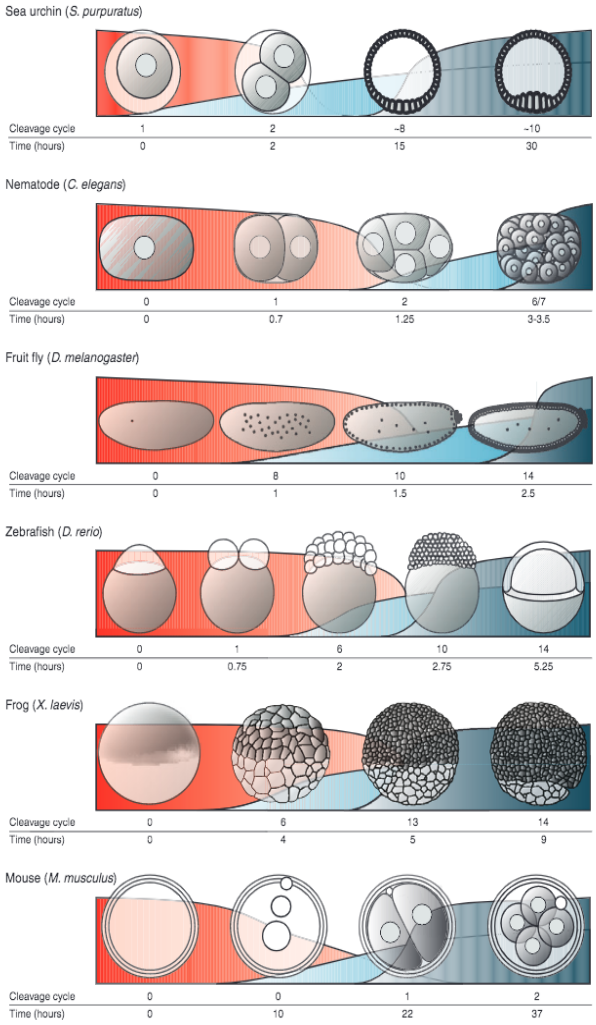

Fig. 1. A comparative overview of the maternal-to-zygotic transition (MZT) in several model organisms. Key embryonic stages for each model organism are depicted schematically above the corresponding cleavage cycle and time after fertilization. The red curves represent the degradation profiles of destabilized maternal transcripts in each species. The light and dark blue curves illustrate the minor and major waves, respectively, of zygotic genome activation. The last embryonic stage presented for each organism is the developmental point at which there is a major requirement for zygotic transcripts.

Figure 2. The maternal-to-zygotic transition across model organisms

This diagram demonstrates how the maternal-to-zygotic transition occurs across different animals during early embryonic development, with the red curve demonstrating the degradation of maternal mRNAs and proteins, and the blue curves demonstrating the process of embryonic genome activation. (Tadros & Lipshitz, 2009).

It is possible that over the course of the maternal-to-zygotic transition, several errors may occur which could result in hybrid embryo mortality. For example, there could be issues in the process of the mother implanting mRNAs and proteins into the egg, issues in the embryo translating maternal mRNAs, or issues in the transition from translating maternal mRNAs to transcribing embryonic genetic material, for example.

Comparison to Polyploidy in Plants

While polyploidy is relatively rare and often lethal within animals, especially in birds and mammals, it has a very high incidence in organisms such as plants and fungi. In some instances where polyploidy is survivable in vertebrates, this phenomenon may render individuals sterile due to the inability for chromosomes to properly pair during the division of sexual cells (Frazer, 2013). In plants, however, polyploidy can serve evolutionary benefits by creating more variability within a gene pool. For example, this could lead to a diversification in gene function; additional copies of genes may be used in other ways over time, evidenced by drastically varying gene expression patterns among polyploid plants of the same species (Adams & Wendel, 2005). This, coupled with their significantly flexible and ultimately forgiving body plans, has allowed for an extensive survival of polyploid plants (Frazer, 2013). It has been estimated that two thirds of angiosperms exhibit polyploidy, including over 99% of ferns and

80% of grass family species such as wheat, oats, corn, and rice, as well as other crops including potatoes, apples, and sugarcane (Frazer, 2013).

In general, crops have been artificially selected to be polyploid due to the greater yield that this provides. Some polyploid vertebrates, such as triploid trout, see a significant increase in size in comparison to their diploid counterparts, although these trout are consistently sterile (Frazer, 2013). Not only are polyploid organisms larger in size due to the increased amount of DNA that their cells are required to contain, but also because this increased amount of DNA has been postulated to lead to a greater amount of gene product generation (Frazer, 2013). This leads to not only larger harvests, but also plants which are more robust, as polyploidy often serves as a measure to protect plants against prejudicial recessive mutations.

Unlike polyploidy in some animals such as the triploid trout, *Ambystoma* hybrids are capable of producing offspring, with only about 1% being sterile (Lowcock et al., 1991). Similarly to the triploid trout, however, hybrids ranging from triploid to pentaploid are significantly larger than diploid and haploid hybrids (Lowcock et al., 1994). Whether this is due to cells becoming enlarged due to the increased amount of genetic material they are required to contain, or an increased amount of transcription and translation occuring in hybrids with higher ploidies, is currently unknown.

Salamander Genomomics

Genomomics is the study of the composition and construction of a genome, with the genomomic makeup of an individual being referred to as its genomotype. Genomotype refers not only to how many copies of chromosomes are present in a polyploid individual's genome, but also, of which species they originate from in the case of hybrids. In the literature, *Ambystoma laterale-jeffersonianum* complex hybrids' genomes from *A. laterale* are abbreviated as L and their genomes from *A. jeffersonianum* are abbreviated as J. For example, a triploid hybrid with one copy of *A. laterale* chromosomes and two copies of *A. jeffersonianum* chromosomes would be referred to as having the genomotype LJJ. In general, several differences between *Ambystoma* hybrids have been recorded, contributing to the theory that genomomics may play a significant role in hybrid embryo mortality (Lowcock et al., 1994).

These differences in genomotypes appear to have a substantial effect on the development of hybrids, as LJJ and LJJJ genomotype hybrids grow significantly larger than their JJ genomotype counterparts despite possessing genes from *A. laterale*, a species which is generally much smaller (Lowcock et al., 1994). This may suggest a compounding effect of certain growth genes, incorporating all genomes into transcription and subsequent protein translation. While this is partially due to differences in breeding seasons, which creates differences in size that are correlated with changes in temperature, this alone does

not completely explain this phenomenon, suggesting a codominance of genes influencing structural composition and regulatory processes during development. This explains why LLLJ hybrids are more similar to *A. laterale* diploids than they are to LLL triploids (Lowcock et al., 1994).

Furthermore, within the research completed by Charney et al. in their 2019 study, it was found that across ten sites of embryo collection, the majority of egg masses were found to have the genomotype JJ, LJJJ, and LJJ. In addition, there were also some LJ and LLJ egg masses observed, although these were significantly more rare. Of these, the only true sexual JJ egg masses had a mortality rate of about 10%, whereas LJ, LJJ, and LJJJ hybrid egg masses all had mortality rates which approached 40% (Charney et al., 2019). LLJ hybrid egg masses had a mortality rate of about 20%, although only two LLJ egg masses were found (Charney et al., 2019). Due to the wide range of LJ, LJJ, and LJJJ egg mass mortality, and especially considering that many of these egg masses had mortality rates between 40% and 100% while JJ egg mass mortality peaks at about 30% excluding one outlier, it is reasonable to suggest that genomotype, and hybridity in general, may increase the risk of mortality during embryonic development (Charney et al., 2019).

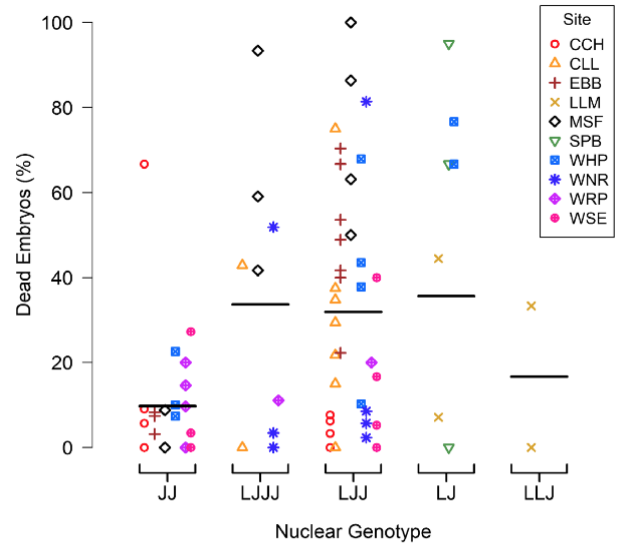

Fig. 2 Observed frequency of dead embryos in field surveys at 10 sites across Massachusetts. Each point represents a whole egg mass, with genetic identity inferred by microsatellite markers from a single embryo taken from the egg mass, whereas nuclear genotypes are known to vary amongst embryos in a mass. The horizontal lines represent the mean percent dead embryos among masses within each genotype group. In describing nuclear genotypes, the number of times each letter is repeated represents the number of copies of each genome set, with "L" for *Ambystoma laterale* and "J" for *A. jeffersonianum*

Figure 3. Genomotypes and mortality of *Ambystoma* complex samples
This figure illustrates the mortality of different genomotypes of *Ambystoma* hybrid embryos collected from across Massachusetts. The majority of samples collected had the genomotype JJ, LJJJ, and LJJ, with LJJJ, LJJ, and LJ having the highest rates of mortality. (Charney et al., 2019)

Genetics Prior to First Cleavage

Following fertilization, the genome of an organism is transcriptionally inactive. At this point, the embryo relies on mRNAs and proteins provided by the mother until the maternal-to-zygotic transition, which brings about embryonic genome activation. This process of embryonic genome activation first undergoes its minor phrase, in which it experiences more minimal transcriptional activity and does not utilize transcription factors. Next, it enters its major activation phase, in which transcription occurs at a more rapid rate through the use of transcription

factors; much of what is synthesized in this major activation phase is crucial for the development of the embryo. Embryonic genome activation is reliant on the activity and availability of basal (general) transcription machinery, as well as on the physical modifications that embryonic nuclei undergo post-fertilization (Jeanblanc et al., 2008). Because no transcriptional activity occurs in the earliest stages of embryonic development, we must look to the influence of maternally-provided mRNAs and proteins, as well as other genetic factors, in order to examine how embryos may be influenced around the event of first cleavage.

More specifically, we must examine, on a molecular and genetic level, what is occurring within the embryo around the 1-cell stage and the 2-cell stage, as it has been suggested that hybrid embryo mortality occurs around the time of first cleavage (Avis, 2019; Charney et al., 2019). Given our understanding that the embryonic genome is activated in a stepwise manner, it may be reasonable to assume that the amount of genes and gene products which are active during these stages of development are considerably fewer than those which are active later on, substantially minimizing the amount of factors we must consider in attempting to determine the factors at play that may result in *Ambystoma* hybrid embryo mortality.

The 1-cell stage zygotic development can be divided into two parts, early and late, where the early 1-cell stage zygotic cytoplasm does not support a significant degree of transcription, and none is supported from exogenous nuclei

or genetic material, whereas in the late 1-cell stage embryo, transcription from exogenous nuclei is supported. This allows for the transcription of transgenes, as well as of some genes which are specific to the 2-cell stage, when these genes are experimentally implanted in the late 1-cell stage zygote (Latham & Schultz, 2001). Experiments leading to such conclusions suggest that genome activation, at least in the case of the mouse, is not a singular event, reaching major milestones at the late 1-cell stage, the early 2-cell stage, the late 2-cell stage, and the 8-cell stage (Latham & Shultz, 2001).

Another crucial protein which plays a role in early genetics is the somatic form of the histone H1 protein. This has been found to be expressed not only at the 4-cell and 2-cell stages, but also at the 1-cell stage. Within the 1-cell mouse embryo, it has been found that synthesis of histone H1 is driven by mRNAs that are maternally encoded, and that this synthesis declines briefly at the 2-cell stage before increasing again at the 4-cell stage, in line with the maternal-to-zygotic transition (Latham & Schultz, 2001). When somatic histone H1 is microinjected into early stage embryos, it joins with DNA to form chromatin. Additionally, synthesis of histones H2A and H3 are also increased at the 1-cell and 2-cell stages; it has been found, however, that while synthesis of histones H1 and H4 is a wholly result of translation of maternal mRNAs, some expression of histones H2A and H3 can be attributed to expression by the embryonic genome. While maternally encoded mRNAs are not responsible for the entirety of histone

synthesis during the earliest stages of embryonic development, they do code for the vast majority, meaning that they are entirely necessary for the generation of a sufficient amount of histones (Latham & Schultz, 2001).

Here, issues regarding the maternal-to-zygotic transition in relation to histones could also be partially responsible for *Ambystoma* hybrid embryo death. For example, a lack of proper synthesis of any of the histone octamers necessary for forming chromatin may result in the improper transcription later on in the embryo. In one study surrounding early *Ambystoma* hybrid embryo mortality found that hybrid embryos contained large amounts of uncondensed chromatin (Elinson et al., 1992). If histones are being generated in excess in *Ambystoma* hybrid embryos due to either issues regarding degradation of maternal mRNAs or upregulation of the entire genome, it is possible that this could explain the large amounts of chromatin found in some dead embryos.

SECTION 3: ENVIRONMENTAL CONCERNS

Impact of Water Quality on Amphibian Development

Populations of *Ambystoma* embryos are left extremely vulnerable to the elements due to the nature of amphibian eggs. In the case of many species of the *Ambystoma* genus, such as *Ambystoma maculatum*, eggs are laid coated in a layer of jelly as a protective measure (Gomez-Mestre et al., 2006). This method is also utilized by unisexual *Ambystoma* females (Bogart et al., 2016). Even with this protective layer, however, embryos are not wholly protected, being exposed to several external factors that may be influential regarding their survival or mortality.

One factor to consider in future research is that the hybrid embryos are oviposited in vernal pools, which are seasonal bodies of water formed from rain and snowmelt in wooded areas across the North American northeast. Data from these pools must be obtained when the embryos are collected for further research. Several measures, such as salinity, pH, and temperature must be taken from these vernal pools in order to determine which factors may be contributing to embryo death.

Figure 4. A vernal pool in the state of Massachusetts
A typical vernal pool in New England. (Commonwealth of Massachusetts, 2021).

Salinity, for example, could play a major role in embryo death, as North American amphibians are intolerant to salt and typically only inhabit freshwater aquatic systems (Meindl et al., 2019). This is due in large part to the permeability of amphibians' eggs and skin as adults, which relies on freshwater. Because vernal pools are created by snowmelt, this water could likely be saturated with dissolved rock salt if pools are formed anywhere near a road. This rock salt is composed of the same salt which is found in table salt, as well as in bodies of water with high salinity such as oceans, meaning that this salt could be equally as harmful to *Ambystoma* hybrid embryos as naturally occurring saltwater (United States Geological Survey, 2015). Road salt itself has also been found to be extremely detrimental to the development and survival of amphibians such as *Lithobates pipiens* and *Xenopus laevis* (Meindl et al., 2019). Because most amphibians

struggle to thrive in even brackish water environments due to its dehydrating nature, the salinity of vernal pools should be less than 1,000 parts per million of dissolved salts (United States Geological Survey, 2015). If clutches of *Ambystoma* hybrid embryos are being affected by water with high salinity, it is possible that embryos on the outer layers would be exposed and die, while the inner embryos would be protected.

In terms of pH, basicity is ideal in comparison to acidity, as low pH can stunt growth of amphibian embryos and has also been found to lead to increased embryo death (Horne & Dunson, 1995). Not only is the survival of *A. jeffersonianum* embryos in the presence of basic water high, but it remains high in the presence of non-toxic metals. Living in basic water in the presence of toxic metals, such as aluminium and copper, however, presents a relatively high mortality rate. Additionally, exposure to toxic metals was found to be more survivable in more acidic waters (Horne & Dunson, 1995). Out of these combinations, it is possible that *Ambystoma* hybrid embryos in nature could be exposed to either acidic waters, or to basic waters and toxic metals. This could occur in instances such as acid rain being generated from sulfur dioxide and nitrogen oxides from pollution accumulating in the atmosphere and being rained down across New England, or from minerals or copper pipes in the earth breaking down over time, being leached out of the ground during the formation of vernal pools.

Temperature also plays a significant role in *Ambystoma* salamander development and survival. For example, *A. jeffersonianum* eggs laid in waters which are maintained at 21ºC hatch in about two weeks, whereas those laid in water of temperatures consistent with the typical weather of late winter and early spring can take up to 14 weeks to hatch (Kipp, 2000). Because lower temperatures are correlated with slower development, *Ambystoma* hybrid eggs laid at the beginning of the season experience development more slowly. In this case, stages in development such as the first cleavage event likely take more time to occur, increasing the amount of time for outside influences to interfere with development at a given stage. Additionally, it has been found that temperature has profound effects on *Ambystoma* reproduction. At lower temperatures, *Ambystoma* females most frequently reproduce via gynogenesis, and at higher temperatures, hybridogenesis is favored (Bogart et al., 1989). Because of this, hybrid eggs are most frequently laid at the end of the season.

Effects of Pollutants and Fungi on Development

It is plausible that during the course of the life of the vernal pool, minerals, metals, and sediment will leach from the soil into the water. Especially considering that *Ambystoma* complex salamanders spend a minimum of four months in vernal pools prior to their metamorphosis, anything in the water can permeate the egg or skin with relative ease (Massachusetts Division of Fisheries

and Wildlife, 2016). Though no definitive studies have been completed observing different sediments' effects on *Ambystoma* hybrid embryos, many studies have been completed using related species.

For example, one study found that at high concentrations, cadmium leads to embryonic limb degeneration and had a 50% mortality rate in *Ambystoma mexicanum* and *Ambystoma gracile* populations after 96 hours, suggesting that this may be a possible contributor under some circumstances, but would likely not be the only factor promoting mortality in embryos prior to first cleavage (Naval Facilities Engineering Command, 2004). Because the first cleavage event of these hybrid embryos occurs a mere hours following oviposition, I mainly focus here on studies which have found various metals, pollutants, and organic materials to be lethal soon after exposure to embryos.

Sediments which are most likely to cause embryo death, given what we know about hybrid *Ambystoma* embryo cellular division, are copper, mercury, and zinc (Naval Facilities Engineering Command, 2004). Copper exposure has a 50% embryonic mortality rate of *A. jeffersonianum* within 96 hours. It can be found in rocks, minerals, and clays in the earth, as well as in plumbing, electrical wiring, insecticides, and other man made products. While copper is a necessary metal for growth and metabolic function in small amounts, even minor increases have been found to be lethal to developing amphibians (Naval Facilities Engineering Command, 2004). However, these effects tend to vary between clutches of eggs,

with larger clutches having lower rates of mortality than smaller clutches. Additionally, while it is currently unknown exactly why an excess of copper is lethal, it has been hypothesized that this is partially caused by copper ions inhibiting the diffusion of oxygen across amphibian egg membranes (Soteropoulos et al., 2014).

Additionally, mercury exposure has a 50% mortality rate in *A. mexicanum* embryos after both 24 and 48 hours. Mercury is found in various rocks and ores, and fossil fuels such as crude petroleum and coal. Around 80% of mercury deposited into the environment anthropogenically originates from fossil fuel combustion, smelting, waste incineration, and mining; with pollution having such devastating ecological effects, an excess of mercury in the environment and in water sources calls attention to the importance of movements away from the use of fossil fuels on an industrial level (Naval Facilities Engineering Command, 2004). It has been hypothesized that maternal exposure to mercury may result in additional compounds being transferred into the egg, ultimately becoming lethal very early on in embryonic development, although mechanisms which could cause this are currently unknown (Bergeron et al., 2010).

Zinc exposure has a 50% mortality rate in *A. opacum* embryos after 24, 48, and 96 hours. Zinc is present in several minerals within the earth's crust, but human activity is responsible for over 96% of the total amount of zinc in the environment; it is used in batteries, cable wrappings, alloys, and auto parts,

among many other products and processes. It is also used as a fungicide, herbicide, and rodenticide, as well as in cosmetics, glass, glue, ceramics, inks, and plumbing. While low amounts are necessary for regulating metabolism, zinc toxicity is common and is caused through direct exposure from dissolved zinc in bodies of water. (Naval Facilities Engineering Command, 2004). It has been found that zinc can stimulate development and growth in the Chiricahua leopard frog *Lithobates chiricahuensis*, meaning that lethality may be due to this rapid growth or related errors in genome replication (Calfee & Little, 2017).

Another possible contributor may be polycyclic aromatic hydrocarbons (PAHs), chemicals occurring naturally in crude oil, coal, and gasoline, which are often present in products composed of creosote, coal-tar pitch, and asphalt (Illinois Department of Public Health, 2021). PAHs have been found to cause stunted growth in *A. maculatum*, as well as a 5-10% mortality rate upon exposure (Naval Facilities Engineering Command, 2004). While PAHs may not necessarily be responsible for a large portion of *Ambystoma* complex hybrid embryo deaths, it could be responsible for slowing cleavage events, allowing other chemicals or biological factors to kill embryos prior to their first cleavage events. In general, PAHs are known to mimic steroid hormones such as estrogen in mammals, and have been known to cause medical issues such as breast and reproductive tract cancers in humans, indicating that this could also have profoundly detrimental effects in *Ambystoma* development (Santodonato, 1997). Other compounds with

high estrogenic activity, such as 17α-ethinylestradiol, have been found to disrupt mating behaviour such as mate calling in *Xenopus*, and deterring mating behavior in female *Xenopus*, suggesting that PAHs might have a similar effect on *Ambystoma*, possibly contributing to their population decline as a whole (Hoffmann & Kloas, 2012).

In terms of pesticides, there has been no research conducted on *Ambystoma* amphibian embryos' relationship with dichlorodiphenyltrichloroethane (DDT), dichlorodiphenyldichloroethane (DDD), or dichlorodiphenyldichloroethylene (DDE). However, they have generally been known to cause deformities, impaired development, and have a 50% mortality rate in reptile, amphibian, and bird embryos within 24 hours of exposure (Naval Facilities Engineering Command, 2004). Because the use of all three of these pesticides is heavily advised against, its presence in vernal pools and the surrounding areas is unlikely, especially considering that they have a halflife of only between 2 and 15 years (National Pesticide Information Center, 1999).

Another category of pesticides are organophosphorus pesticides (OPPs), including chlorpyrifos and malathion, which have been found to have somewhat lethal in early *A. mexicanum* embryos in varying concentrations over all periods of time considered. The majority of the lethality of malathion is found in the early larval stages whereas the majority of lethality of chlorpyrifos occurs during the mid to late embryonic stages of development (Robles-Mendoza et al., 2009).

However, malathion has been found to significantly prolong embryonic development, suggesting that while it may not necessarily be lethal in early embryonic development, it may lengthen the earliest stages of embryonic development, thereby increasing the length of time during which specific events like first cleavage occur (Robles-Mendoza et al., 2009). This supports the theory that because embryonic development may become drawn out due to external factors, there is a greater period of time in which other factors may result in mortality. This slowing of development also complicates *Ambystoma* salamander survival because while embryos developing in water that contains malathion do not die, their transference into clean water has been found to kill them within 24 hours (Robles-Mendoza et al., 2009). If *Ambystoma* embryos are found in vernal pools containing malathion or similar compounds in nature, they cannot be transferred into different water, meaning that alternate methods of protection may be necessary.

Additionally, fungi such as *Batrachochytrium dendrobatidis* have been known to have devastating effects on amphibian populations across the world, with one amphibian host being *A. jeffersonianum*, as well as other species such as *A. opacum* and *A. mexicanum* (Venesky et al., 2010). It has been found that *B. dendrobatidis* is able to live and grow in soil, providing a basis for the logic that this fungus may be growing in the soil where vernal pools form or in the surrounding areas, leaching out of the soil and infecting populations of

Ambystoma females, as well as *A. jeffersonianum* and *A. laterale* (Venesky et al., 2010). This pathogen is a significant threat to amphibian populations such as the *A. laterale-jeffersonianum* complex because it is the disease-causing agent of Chytridiomycosis, which can cause dramatic changes in osmotic regulation that can trigger cardiac arrest in adult salamanders. Other effects include epidermal hyperplasia, hyperketosis, and ulcers (Venesky et al., 2010).

Through an exposure experiment, it was found that *B. dendrobatidis* had minimal effects on the larval development of *A. opacum*, although it was noted that it may have impacts of different severities based on when in development infection occurs. The salamanders tested in this experiment were of a late larval stage, meaning that there is a possibility that *B. dendrobatidis* could have different effects on earlier larval stages, or on *Ambystoma* species embryos (Venesky et al., 2010). Especially considering its ability to disrupt osmotic regulation in late larval stages and adults, it is possible that *B. dendrobatidis* infections occurring in the earliest stages of development may cause cells to lyse, killing embryos.

Possible Impact of Magnetic Fields on Development

Another factor which could contribute to *Ambystoma* complex hybrid embryo survival is pulse magnetization, which has been noted to affect gene expression in the central nervous system of the spiny lobster *Panulirus argus*. In these lobsters, it was found that pulse magnetization prompted an increase in gene

expression of over 10% of all expressed genes which were affected. Several of these genes are involved with DNA damage and repair, supporting a theory that a similar phenomenon may be occurring in *Ambystoma* hybrid embryos during development. This phenomenon is hypothesized to be caused as a result of *P. argus*' consumption of magnetite, a magnetic mineral, which is thought to be ingested over the course of the lobster's life (Ernst et al., 2020).

Although *Ambystoma* salamanders typically only consume insects and other invertebrates, likely not consuming pieces of metallic mineral in the same manner as *P. argus*, their permeable eggs and vulnerable skin makes it possible that magnetic metals in the soil or water of vernal pools may have a similar effect. Of the possible metals and other materials which could be causing *Ambystoma* hybrid embryo mortality, aluminium is magnetic. Additionally, though zinc itself is not magnetic, it possesses many of the properties of magnetic materials and can interact with magnetic fields, making it possible that the absorption of zinc could cause mortality via magnetic pulses originating from urbanized areas or heavy machinery.

Concerns Regarding Global Warming

Many concerns regarding the *Ambystoma* complex's survival, including concerns surrounding hybrid embryo death, pertain to how global warming may affect breeding and survival. These salamanders reproduce in vernal pools, which

are seasonal bodies of water generated by rain and snowmelt (Massachusetts Division of Fisheries and Wildlife, 2020). As global temperatures continue to rise, there is a likelihood that these vernal pools will not only evaporate prematurely, resulting in the death of many of these embryos, but they may also be formed too early as well. Although global warming is only currently marked as a minor threat to the *Ambystoma laterale-jeffersonianum* complex, it is highly likely that this threat will increase over time (Government of Canada, 2015). This is also compounded by the fact that hybridity is a more frequent phenomenon in warmer temperatures (Bogart et al., 1988). Because increased temperatures would result in a greater proportion of offspring being conceived via hybridogenesis, which have a significantly higher rate of mortality in embryonic development than nonhybrids, this may lead to a significant drop in the *Ambystoma* complex population.

Additionally, one study analyzing the impact of climate change on *A. maculatum* found that as temperatures increase each winter, migrations to breeding sites also occur increasingly earlier (Kirk et al., 2019). Though this does suggest that salamanders of the *A. laterale-jeffersonianum* complex may similarly be adapting to global warming, other additional factors were found which could cause complications in other areas of the salamanders' ecology. Other factors which have been found to correlate with climate change in *A. maculatum* include increased body size, disease vulnerability, and general population declines (Kirk

et al., 2019). Not only could the *Ambystoma* complex be threatened by premature evaporation of vernal pools, but increased body size could leave salamanders more vulnerable to predators, and increased vulnerability to disease could result in large swaths of the population being killed relatively instantaneously.

Legal Factors and Conservation Measures

In the event that it is determined that the cause of hybrid *Ambystoma* embryo death is due to unfavorable environmental conditions because of the presence of toxic compounds or to global warming, for example, it may be necessary to institute conservation efforts in order to support the populations of these salamanders.

Because of their threatened and endangered statuses across New England and eastern Canada, several plans have been proposed in order to protect the *Ambystoma laterale-jeffersonianum* complex and its related species. The Government of Canada, in a proposed recovery strategy series of *A. jeffersonianum* under the Species at Risk Act, states that four criteria must be examined to determine the feasibility of recovering threatened populations. It is known in confidence that two of these criteria are met, one which states that individuals of a species are capable of reproducing currently or in the future in order to increase the population, and another which states that recovery

techniques to restore the population count and distribution exist and are reasonably accessible (Government of Canada, 2015).

The two other criteria, one which states that there is sufficient habitat that is or could be available to support the species as it grows, and one that states that threats to the species or habitat can be mitigated or avoided, are currently not known (Government of Canada, 2015). These factors are complicated due to population trends of *A. jeffersonianum* within Ontario being largely unknown, especially because much of the area is dominated by unisexual *Ambystoma* females, which are extremely phenotypically similar. Without the presence of *A. laterale* and *A. jeffersonianum*, however, these populations of unisexuals will not survive. There are also additional complications due to aggregate extraction, urbanization, road construction, and other related activities have been found to take priority over conservation, with many of these processes themselves threatening *Ambystoma* populations mostly by way of habitat loss or habitat contamination (Government of Canada, 2015).

Because the conservation biology surrounding *A. jeffersonianum* in the Ontario area is well known, specific sets of recommended regulations for habitat regulation have been published, detailing how to protect this species and its related complex. Currently, the Canadian government recommends that all wetlands and surrounding wetland features that are suitable for breeding be maintained. It also recommends that open terrestrial areas of *Ambystoma* complex

habitat areas within 300 meters (984.25 feet) be maintained and that conditions in these areas allow for migration, dispersal, foraging, and hibernation. It is also suggested that corridors be implemented in order to make connections between breeding locations, given that they are less than one kilometer (0.62 miles) away (Government of Canada, 2015).

As of right now, Canada lists that threats to the *Ambystoma laterale-jeffersonianum* complex may include loss of habitat due to urbanization and mining, the construction and general presence of roads, anthropogenic disturbance, deforestation, recreational use of trails, and unauthorized collection and introduction of carnivorous fish (Government of Canada, 2015). It is also noted, however, that there are many gaps in our knowledge which complicate many conservation efforts, such as the exact distribution of *A. jeffersonianum* and unisexual *Ambystoma* salamanders and dispersal patterns during fall migrations (Government of Canada, 2015).

Within the state of New Hampshire and across several other states within the United States, the *Ambystoma laterale-jeffersonianum* complex is indirectly managed and protected via forestry management, land preservation, and wetland and water resource protection regulations, meaning that there are no specific legal measures designed to protect these populations of salamanders. According to the New Hampshire Wildlife Action Plan, the greatest threats to the *Ambystoma* complex populations within the state are residential and commercial development,

filling of wetlands, vehicles, and acid deposition. Lower ranking threats include the use of fertilizer near wetlands, invasive plants, and droughts (New Hampshire Fish and Game, 2015). This action plan includes maintenance of habitats and dispersal corridors, as well as extensive surveys to study size and genotype of individual salamanders, and their proximities to roads and land development areas, ultimately noting that more data on this population and its distribution is necessary before specific conservation plans may be made (New Hampshire Fish and Game, 2015).

There are also objectives to distribute information either on a state level, from the government and non-profit groups, or on the municipal level via more localized organizations. This would take the form of volunteers educating community members on *A. jeffersonianum* and the *Ambystoma laterale-jeffersonianum* complex in general, teaching individuals how conservation measures are being instituted and what can be done on an individual level to keep salamanders safe. This would include information regarding when migrations to vernal pools occur, instructing New Hampshire residents to be cautious of salamanders crossing roads (New Hampshire Fish and Game, 2015).

Similarly in the state of Massachusetts, vernal pools that *Ambystoma* complex salamanders utilize for reproduction are protected under Title 5 of the Massachusetts Environmental Act, Section 401 of the Federal Clean Water Act, the Massachusetts Surface Water Quality Standards relating to Section 401, and

the Massachusetts Forest Cutting Practices Act (Commonwealth of Massachusetts, 2021). Specific recommendations to protect vernal pools include an advised buffer zone of a 123 foot radius surrounding vernal pools and at least a 50% crown cover retention, meaning that at least 50% of this area should be protected via the crowns of surrounding trees. Some have suggested a zone of intensive habitat protection between 534 and 574 feet from vernal pools, with some species such as *Ambystoma cingulatum* being recommended to have 1,476 feet of buffer zone (Massachusetts Division of Fisheries and Wildlife, 2007).

Within Massachusetts, an extensive array of both required and recommended practices for wildlife management exists in order to protect *Ambystoma* complex salamanders and their habitat. The following table details this list of forestry conservation management practices:

Table 1: Required and recommended land management of vernal pools

Required Management Practices	Recommended Management Practices
■ No harvesting may occur in any vernal pools listed within the Ambystoma habitat. ■ Retain an undisturbed filter strip between 0 and 50 feet from vernal pools.	■ Extending the 450-foot radius to 600 feet is ideal but not necessary ■ More than 70% of the 450-foot radius surrounding the vernal pool could have a 75% canopy cover ■ When vernal pools are clustered, new installments or maintenance should not be located between them.

■ Retain a 70% or greater canopy cover over 65% of the area of land between 50 and 450 feet from vernal pools. (This canopy cover cannot be concentrated around the vernal pool.) ■ If harvesting must be done within the 450-foot radius, a licensed forester must draw up a detailed cutting plan including how the area will meet requirements for salamander habitats following the harvest. ■ If the 450-foot radius is left uncut during a harvest, a licensed forester is not required to create a cutting plan. ■ New areas of pavement must be made at least 100 feet away from vernal pools. ■ Use of motorized vehicles between 50 and 450 feet from vernal pools may only only be done between May 15th and February 28th in areas where A. jeffersonianum is native, and between October 15th and August 15th in areas where A. opacum is native.	■ At least two snags per acre should be left standing in order to provide debris to serve as shelter. ■ Leave sections of wood which are at least 12 inches in diameter and 15 inches long to provide shelter or microhabitat. ■ Do not disturb fallen logs. ■ Leave tree trimmings in the forest where they are cut. ■ Harvest during the winter in order to avoid compacting soil and disturbances to the forest floor.

(adapted from Massachusetts Division of Fisheries and Wildlife, 2016)

In general, many of the available government documents detail the current status of *A. jeffersonianum*, *A. laterale*, and *Ambystoma* unisexuals and hybrids, as well as proposed and current measures put in place to protect them, while little

data appears to be available that discusses the results of these practices. Much of this is likely due to the small amount of data regarding these salamander populations in general. Because of this lack of data, along with the emphasis of general environmental preservation rather than concern for *Ambystoma* species and specific populations of these species, it is certain that more research must be completed and more measures must be put in place to protect the *Ambystoma* complex across North America.

While many threats to these populations such as vehicles and urbanization are extremely feasible to combat on a bureaucratic level, additional threats to embryos which contribute to early hybrid embryo mortality may be at play, stifling population growth. In addition to the continuation of protection from a legal standpoint and public education, research into the waters and soils of vernal pools, as well as investigations into illegal filling of vernal pools must be done in order to protect the *Ambystoma laterale-jeffersonianum* complex and its related hybrids.

SECTION 4: FUTURE DIRECTIONS

Routes for Future Study

Due to the myriad of explanations as to what could be causing premature hybrid embryo death, there are a profound amount of avenues for future study in biological and environmental contexts, taking the forms of both laboratory research as well as field work. On a general level, these experiments could take the form of genetic and environmental analysis, examining factors such as gene expression, chromosomal quantification, and water quality testing in order to deduce exactly what is responsible for early hybrid embryo death.

Going the route of testing specific genes and proteins such as maternal VegT and EP-cadherin, one method that could be implemented is one-step reverse transcription quantitative polymerase chain reaction, or RT-qPCR. This technique can be used in genetic research in order to measure the level of gene expression based on RNA, such as mRNA, being transcribed (Thermofisher Scientific, 2021). Once this RNA is isolated, it can then be converted into a first-strand cDNA via a transcriptase enzyme. Using this cDNA as a template, qPCR could then be performed through introducing primers specific to the VegT or EP-

cadherin genes, for example. The PCR reaction could be done in the presence of a nucleic acid stain like SYBR Green, which will bind to double-stranded cDNA molecules and fluoresce. As more double-stranded PCR product is generated, the more fluorescence is produced. After multiple rounds of the PCR cycle, the fluorescence will reach its cycle threshold; the more gene-specific RNA was in the original sample, the lower the cycle threshold. Because of the nature of RT-qPCR, it would be necessary to run a comparison between diploid *A. laterale* or *A. jeffersonianum* embryos at around the time of first cleavage and compare this against hybrid embryos at the same stage of development. From here, the cycle thresholds could be compared, allowing us to quantify whether specific genes are being expressed at higher or lower rates in hybrids than in the *Ambystoma* sexual populations.

RT-qPCR may also be useful for measuring the process of the maternal-to-zygotic transition. Here, it instead could be used to measure quantities of maternal mRNAs and compare them against embryonic mRNAs via their cycle thresholds. A baseline of maternal and embryonic mRNA can be obtained via analyzing the cycle thresholds of diploid *A. jeffersonianum* and *A. laterale* embryos. This could then be compared against the cycle thresholds of diploid LJ *Ambystoma* hybrid embryos to establish if similar amounts of mRNAs are both provided by the mother and being produced by the embryo following the maternal-to-zygotic

transition. These cycle thresholds could then be compared against hybrids of other ploidies and genomotypes.

In examining errors which have occurred during the maternal-to-zygotic transition, as well as genomotypic differences, techniques such as RNA-Seq could be employed. This is a technique used to quantify the amount of RNA present in a sample, which correlates to the amount of transcription occurring in a sample. In RNA-seq, cDNA libraries can be generated from cells of a given sample. From there, RNA can be sequenced and converted to cDNA. Then, linkers which are necessary for sequencing cDNA can be added; finally each cDNA molecule is sequenced and counted. The amount of gene expression can then be determined based on the amount of individual cDNAs of a certain sequence present in a sample. This will provide an all-encompassing perspective on the transcription and translation of all maternal and embryonic mRNAs present in the *Ambystoma* hybrid embryo in its earliest stages.

Studying the regulation of gene products, the use of RNA-Seq would also be beneficial. Here, it could be used to quantify and compare the total amount of mRNAs present in early *A. jeffersonianum* and *A. laterale* embryos against those of unisexual *Ambystoma* embryos, diploid LJ hybrid embryos, and hybrids of greater ploidies and varying genonmotypes. This will assist in determining whether gene product is regulated and therefore uniform across all ploidies and genomotypes, or if this varies and increases with ploidy. This data may then be

analyzed while considering which of these embryos were found dead and which were found living in order to formulate more specific hypotheses regarding mortality and ploidy or mortality and genomotype.

To test specifically for aneuploidy, it may also be necessary to perform karyotyping. Karyotyping is a process which entails photography of chromosomes for the purpose of detecting chromosomal abnormalities (Gilbert, 2010). Cell samples from karyotypes must be taken as cells are dividing, as this is the only period of time at which genetic material is condensed into chromosomes. From here, they may be photographed, printed, cut out, and paired together. Not only will this allow us to determine the ploidy of an organism, but conditions such as aneuploidy can also be diagnosed (Gilbert, 2010).

In examining water quality, it will be appropriate to run basic tests to analyze the salinity, pH, and temperature of vernal pools from which *Ambystoma* hybrid embryos are collected. Salinity can be measured through measuring the conductivity of water with a probe and a meter, as conductivity is directly positively correlated with salinity; because conductivity is also positively correlated with temperature, the data taken from this equipment is extrapolated and calibrated for a standard temperature of 25ºC (Environmental Protection Agency, 2006). pH can also be assessed relatively easily, with a potentiometer, which measures the electric potential across the meter's electrode when it is immersed in water. This test can be run in the field or in a laboratory setting, but

if it is completed in a laboratory setting, this must be done within two hours of collection; waiting longer will allow for significant amounts of carbon dioxide dissolving into the water, neutralizing the pH (Environmental Protection Agency, 1992). The temperature of the surface of a body of water may be measured through the use of an infrared thermometer, and the temperature of the body of water in general can be collected through the use of a standard electronic thermometer. In methods of temperature collection it is beneficial to collect data from both areas of shade and sun, and wherever egg clutches are collected from.

In order to detect specific pollutants in the water, it may be possible to employ equipment such as biosensors or vibrational spectroscopy instruments. These tools are capable of detecting anionic compounds in water samples from aquatic environments. These instruments are typically used to perform tasks such as detecting arsenic, toxic metals, bacteria, algae, and yeast in water, and many similar spectroscopy instruments are also capable of making similar assessments of compounds in soil (Zulkifli et al., 2018). This is an advanced form of technology, with some models being miniaturized and even portable. Technologies such as these have revolutionized water monitoring, allowing us to detect an array of possibly harmful compounds and biological matter in bodies of water (Zulkifli et al., 2018). In the context of *Ambystoma* research, this equipment could be used to detect various toxic metals and other detrimental compounds which may be present in the soil and water of vernal pools.

Specific pathogens which may be impacting populations can be detected through methods such as PCR assays, a technique used by Venesky et al. in order to determine whether *D. dendrobatidis* was present in their samples. In order to run this assay, specific regions of DNA within the genome of the specific pathogen must be known, and primers must be synthesized to correspond with DNA sequences which are unique to the pathogen. Two primers must be used, one corresponding to the sense strand and one corresponding to the antisense strand of DNA. The primers must then be mixed with a buffer solution of dNTPs, template DNA, a DNA polymerase such as Taq polymerase, and magnesium chloride. Next, a sample taken from a clutch of eggs being studied, and the target DNA must be extracted. This DNA must be denatured by heating it to about 98ºC, allowing the double stranded DNA to come apart. The temperature is then lowered, allowing the strands of primer to bind to the DNA. Taq DNA polymerase will then extend the primer if the primer has bound to the target strand of DNA. Because there is a much higher amount of primer than there is template complementary strand DNA, the target strand will bind to primers rather than back to its original complementary strand. Once the template strand binds to the primers, the reaction is cooled and then repeated multiple times. The product of this could then be placed in an agarose gel and electrophoresed. Staining this with ethidium bromide and using UV light to visualize the fluorescence of DNA molecules, we would be able to determine whether the pathogen corresponding to

the primers is present; this process would not detect or be skewed by DNA from the *Ambystoma* embryos themselves, as primers are programmed to only bind to and amplify DNA of the pathogen in question. This process is highly versatile and could be used to test for several different pathogens (Venesky et al., 2010).

Implications of *Ambystoma* Research

Although they are of least concern on a global level, *A. laterale*, *A. jeffersonianum*, and *Ambystoma* hybrids are considered threatened or endangered in the majority of the geographic regions that they inhabit, and are of special concern in the state of Massachusetts (Lannoo, 2005). Because it is currently unknown what is causing such a high rate of mortality in the early stages of hybrid embryo development, it is currently impossible to know how to assist these populations, if it is at all possible or reasonable to do so. To some extent, the high rate of hybrid embryo death may also be reflective of embryo death in sexual *A. laterale* and *A. jeffersonianum* populations, especially if hybrids are being largely impacted by their environment. If embryo mortality is determined to be caused by environmental factors, it may also be necessary to study the effects of the environment on sexual *A. laterale* and *A. jeffersonianum* populations more extensively, including populations outside of the region of western Massachusetts.

Through the research of hybrid *Ambystoma* embryonic development and mortality, much will be learned regarding not only the genetics of these

salamanders, but also about the quality of water, soil, forestry practices, land management, and local ecology which supports them. Due to their threatened state, it is imperative that this research take place rapidly and extensively. In the case that *Ambystoma* hybrid embryo mortality is solely a result of genetic factors, this research will provide interesting insight into a unique phenomenon of polyploidy, which is rarely seen in animals; if this is at all influenced by environmental factors, we must use our findings to assist these vulnerable populations of salamanders. My hope for this project is that future students will be able to use the research that I have compiled to design and carry out interesting and scientifically, ecologically valuable research on these salamanders.

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