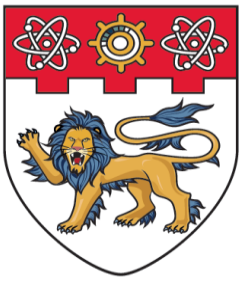

NANYANG TECHNOLOGICAL UNIVERSITY
SINGAPORE

EVALUATING THE EFFECTS OF CALCIUM SUPPLEMENTATION FOR CHICKEN EMBRYOS CULTURED IN A NOVEL BIOMIMETIC TRANSPARENT EGGSHELL

NG SZE XIAN

SCHOOL OF CHEMISTRY, CHEMICAL ENGINEERING AND BIOTECHNOLOGY

2022

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A thesis submitted to the Nanyang Technological University in partial fulfillment of the requirement for the degree of Master of Engineering

2022

Statement of Originality

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Acknowledgments

First and foremost, it is my genuine pleasure to express my tremendous appreciation and gratitude to my primary supervisor, **Professor Teoh Swee Hin**. A passionate senior mentor with teaching excellence, he molded me into a better person and made me understand different guiding principles, which enabled me to work towards my goals and helped me reach where I am today. Without his leadership, kindness, and patience, this dissertation will not have been possible.

I would also like to acknowledge my current supervisor **Professor Sierin Lim**, who was central during the last stretch of my Master's program in ensuring that I can continue reaching my goals by extending a helping hand when in need. This journey will not be possible too without her support and guidance.

In addition, a massive shout out to my fellow friends and mentors, **Dr. Luvita Suryani**, **Dr. Dong Yibing**, **Dr. Charles Lau**, and **Latifah,** for their continual support, advice, and companionship throughout my academic journey. Through them, I understood better the meaning of patience, compassion, and strength of character, which undoubtedly is one of the more significant takeaways of this learning adventure. Thank you for enduring my antics and making the experience more bearable and fun.

Last but not least, my **Mom, Dad,** and partner, **Crystal,** for their unwavering support, care, and concern whenever I encountered any difficulties. They are undoubtedly a part of the backbone of this research work, which came to fruition through their endless encouragement.

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Abstract

The chicken embryo model has long been touted as an attractive alternative for research due to its high growth rate, ease of manipulation, and logistical advantages. There are two primary techniques associated with this model; in-ovo and ex-ovo culture. The in-ovo culture is a method that allows the observation of the embryo through a small window, but poses challenges such as poor visibility, limited embryonic accessibility, and lengthy preparation time. Conversely, the ex-ovo culture functions by cultivating the embryo outside the shell. This addresses some issues highlighted above, but presents new hurdles such as poor viability and sustainability due to a lack of a calcemic eggshell and an unnatural environment. As such, this study aims to optimize ex-ovo culturing for chicken embryos by introducing the usage of a novel biomimetic, transparent polydimethylsiloxane eggshell coupled with calcium supplementation. This polydimethylsiloxane system enables a high degree of optical clarity in a three-dimensional state and retains an elliptical geometry of an egg. Benefits of utilizing such an approach can include enhanced visibility, improved accessibility, as well as overall design robustness. Alongside utilizing eggshell powder as a calcium supplement, the amount and timing of this addition were also investigated to assess optimization strategies. As a result, it was discovered that applying eggshell powder during the earlier embryonic stages was more beneficial in terms of overall growth and viability. Enhanced viability rates were also observed for all groups supplemented with eggshell powder. Additionally, a lower calcium quantity usage was perceived to generate more favorable responses in certain key characteristic aspects. These findings suggest that an extraembryonic calcium supply regulation may be time and quantity-dependent. On this basis, the concept of timing and quantity-based calcium supplementation for chicken ex-ovo models should be considered when designing experimental goals.

Introduction

Background Information

Animal models have long been considered the cornerstone of fundamental and medical research, playing a crucial role in facilitating in-vivo models to validate in-vitro observations. Several significant disciplines, such as drug discovery, cancer research, regenerative medicine, and biomedical studies, heavily depend on these models for progress [1]. Some of these animals can span from larger species such as primates, horses, pigs, and cows, to smaller ones like rodents, rabbits, avian birds, and fish [2, 3]. Amongst these creatures, the chicken embryo stands out as one of the most exciting organisms, having one of the most extended and most distinguished histories for studies in developmental biology. The adoption of this experimental model can be dated as early as 350 BC when Aristotle was reportedly conducting early studies by opening chicken eggs at various developmental stages to study their morphology [1]. In addition, it has caught the attention of philosophers, artists, and aspiring biologists throughout history, who often utilize the chicken embryo to ponder upon fundamental theories such as the essence of life [4]. This trend continued well into the 19^{th} and 20^{th} centuries, where it paved the way for more meaningful discoveries like the diagnosis of the arteries and vein functions and the anatomical growth of organs and structures [5].

Fast forward to the present, the chicken embryo has evolved tremendously as a powerful model system. Over the past few decades, there have been leaps and bounds in technological advances and cultivation techniques, driving rapid progress and accelerating widespread adoption in developmental and molecular biology [4]. Compared to other traditional mammalian models, the chicken embryo possesses several unique characteristics that offer crucial advantages over the rest. In terms of growth, it develops rapidly, undergoing developmental changes (neurulation, gastrulation, histogenesis) within 2 to 3 days of laying and completing this process by the end of 21 days [6]. This is favorable in terms of scalability

when planning high throughput and rapid testing experiments. The chicken embryo is also inexpensive, has high availability worldwide, and generally requires no particular facility for storage or handling [7]. Since embryonic development is highly dependent on the incubation parameters (temperature, humidity) [8], this makes it a highly customizable model as incubation can be terminated at any timepoint to address the corresponding developmental stage for specific experiments.

Another one of its unique distinguishing features is the ease of accessibility to the embryo and the chorioallantoic membrane (CAM). Comprising of a flat, two-layered blastoderm, the embryo is usually located on the yolk's surface [6]. Coupled with the fact that the chicken egg is of sufficient size, this renders it suitable for micromanipulation or time-lapse imaging studies even at early stages [9, 10]. On the other hand, the CAM is a highly vascularized extraembryonic membrane that is primarily responsible for but not limited to gaseous exchange [11]. The robust, functional, and vessel-rich network meant that it could easily be exploited for a wide variety of purposes, including the cultivation of bacteria or vaccines [12], biomaterial studies [2], angiogenic research [13], and even tumor grafting experiments [14]. Furthermore, there is extensive documentation regarding molecular mechanisms in major organ systems [11] and genome sequencing [15], making this model also an attractive choice for transgenesis [16] and epigenetics research [17].

Several cultivation methods have been invented to cater to the various developmental stages of the chicken embryo, namely the in-ovo and ex-ovo cultures. The in-ovo culture is an early technique whereby researchers would create a small window in the eggshell to allow accessibility into the interior before sealing the opening, usually with a transparent film for continued observation [18]. Several papers have expanded upon this in greater detail, concentrating on devising in-ovo variants such as the surrogate model [19, 20] and sealing strategies [21, 22]. This facilitated basic manipulative and imaging studies while allowing

reincubation for further development, which is difficult to achieve in other mammals [6]. However, even though the in-ovo system is functional in terms of the utility provided, this method is not ideal as the small window restricts the embryo's working space and optical visibility [23]. Hence, the ex-ovo culture was introduced. As opposed to in-ovo, the ex-ovo involves cultivating the embryo outside the eggshell, generally on an artificial medium. Some examples can include incorporating Petri Dishes [24], plastic film vessels [25], weigh boats [26], and Polydimethylsiloxane (PDMS) vessels [9]. This alleviates the problems of having poor optical visibility of the embryo and better facilitating experimental methods due to larger working spaces [27]. Hence, the ex-ovo model may be a viable alternative in certain scenarios in terms of greater freedom on embryonic manipulation and the employment of experimental methods.

Despite the advantages of an ex-ovo model system, it has its innate weaknesses. One of the primary concerns with existing ex-ovo cultures is the unnatural environment in which the avian embryo is cultivated. Literature review has proposed that the physical characteristics of the egg do play a crucial role not only in facilitating gaseous exchange or structural protection but also in supporting embryonic development and hatching [28-30]. This unnatural aspect is apparent in early iterations of ex-ovo culture medium designs such as the Petri-Dish itself, which is primarily suited only for early-stage studies due to high mortality and abnormality incidences due to poor nutrient absorption caused by poor nutrient absorption on the flat surface [31, 32]. Indeed, there have been improvements in this situation, with some plastic film and PDMS vessels attempting to reflect the elliptical egg shape as closely as possible [9, 25]. However, challenges pertaining to model sustainability persist, such as factoring in design robustness and supporting egg turning [27]. For instance, plastic film vessels often have to contend with stability, maintaining film surface tension, and potential tearing issues [33]. Furthermore, most current ex-ovo model solutions cannot support egg

turning due to design constraints. Nevertheless, egg turning is critical in avian incubation and correlates with healthy embryonic growth and survivability [34, 35].

The second major concern is the lack of a calcemic eggshell in ex-ovo models. Calcium is a necessary component for avian embryos due to its roles in growth [36], general metabolism [37], and bone development [38]. The chicken embryo derives the majority of this mineral from the eggshell. It has been suggested that the absence of this extraembryonic component can induce a negative influence by calcium deprivation, which contributes to poor growth and increased mortality rates [27, 39]. Several past studies reaffirmed this notion, citing observations of poor viability rates [40], retarded embryonic development [41], smaller whole-body sizes [27], and poorer bone density structures [42]. Consequently, research has also shown that in response to the findings above, the administration of external calcium as supplementation to avian embryos has proven beneficial and can alleviate some of the detrimental effects [40, 43]. Some examples can include calcium carbonate [40], calcium lactate [36], eggshell pieces [43], and even calcium nanoparticles [44]. Nonetheless, there is still work to be done in this space; most implementations suggested by previous research are specific, meaning the supplement parameters are often localized within the study itself. This is because there is no distinct standardization, such as the type of calcium most suitable and the optimal quantity required. Furthermore, different ex-ovo model variants are also often being utilized, making it difficult to generalize.

There is still a need to address the aforementioned primary concerns and contribute to improving the overall viability of avian ex-ovo models for long-term studies and sustainability. As such, this report explores using a novel, biomimetic transparent PDMS eggshell coupled with calcium supplementation as an optimized alternative for the chicken embryo ex-ovo model. Previously, the novelty of this PDMS model was first introduced by Ishak et al., who bioengineered this three-dimensional eggshell to address the lack of 3D optical clarity and as

an experimentation platform for imaging modalities [27]. Compared to other traditional ex-ovo systems, this PDMS model is biomimetic, sustainable, robust, and can support additional features such as egg turning without sacrificing embryonic accessibility or optical visibility [27]. In addition, it was also acknowledged that incorporating external calcium supply will be advantageous in enhancing the viability of the model for late-stage developmental research [27]. Therefore, optimization of this model for this study will be carried out via external supplementation, in which the effects of calcium supply in terms of quantity and period of application will be investigated. In addition, this study also examines the model capabilities by assessing the results of several vital characteristic aspects of embryonic growth development and bone formation throughout the entire experimental period.

Experimental Objective and Specific Aims

The primary goal of this research project is to investigate the effects of external calcium supply on a transparent biomimetic PDMS eggshell as an ex-ovo chicken embryo model for optimization. This will be carried out with the aid of several specific aims, namely, 1) To gain an understanding of external calcium supply on the growth and development of chicken embryos in biomimetic PDMS eggshell. 2) To assess the significance of external calcium supply on overall embryonic bone development of chicken embryos in biomimetic PDMS eggshell. In order to achieve these, the differing calcium quantity supplied (0mg vs. 50mg vs. 100mg) and its application on different embryonic time points (Day 3 vs. Day 10) will be examined.

Literature Review

Chicken Embryo as an Experimental Model

Since the inclusion of avian embryos as a practical model in research, the chicken embryo is favored for its overall advantage in terms of accessibility, flexibility, and ease of manipulation [31]. Compared to other mammalian models, such as rabbits or mice, they possess an inherent advantage in logistics, flexibility, and utility [31, 45]. Essentially, chicken eggs are inexpensive, easy to acquire, and are typically available all year round with minimal supply disruption [46]. Moreover, utilizing chicken embryos obviate any need for a particular storage facility as the eggs can be stored in a cool, dry place for a week before incubatory usage [31]. Doing so provides logistic leverage over other animal models that require special procurement facilities for animal handling before starting any laboratory experiments. Depending on the specific research interest, experimental designs can also be streamlined by incubating the eggs to the targeted developmental stage, enabling the researcher to schedule and plan more efficiently [32].

Due to its versatility, the chicken embryo is often employed in developmental biology [31], anatomical and embryonic studies [47], as well as a vascular model for cardiovascular and cancer research [7]. Approximately two to three days after laying from the mother hen, incubated eggs begin showing signs of initial embryonic development, including gastrulation, somitogenesis, and histogenesis [6]. The embryo then proceeds to form limbs, organs, and complex structural systems before eventually reaching the final maturity stage after 21 days, where it would hatch into a newborn chick [48]. This brief developmental period signifies a much more efficient timeframe for researchers to conduct biological studies efficiently. On the other hand, open access to the chorioallantoic membrane (CAM) is also one of the unique attributes of the chicken embryo model. The CAM is a highly vascularized extraembryonic membrane that serves multiple functions such as gaseous & waste exchange and is vital for the

chicken embryo's survival and well-being [13]. Its vast, interconnected network lays the foundation of a robust and functional platform that can translate into a valuable tool for tissue grafting, tumor kinetics, and angiogenetic studies [13, 46]. In addition, immunocompetence of the chicken embryo only forms around embryonic day 20 due to the incomplete formation of its secondary immune system to derive any innate response [49, 50]. Coupling this information with CAM exploitation certainly highlights adopting a chicken embryo as an ideal candidate for vascular or tumor studies.

The chicken embryo model has also garnered much attention in genetics and epigenetics. Over the years in the genetic space, scientists have compiled an extensive database regarding the chicken genome, allowing them to map about 91% of the entire genome sequence [13, 46]. Surprisingly, it is interesting that there is a strong correlation between humans and the chicken gene [6]. It also extends to conserved sequences spanning from the primary coding region and non-coding intronic and flanking regions [6]. This possibly suggests a high similarity between homologous genes in mammalian and avian species [6].

Regarding epigenetics, the chicken embryo model also presents itself as a highly advantageous system compared to other mammalian species. For example, chickens possess a heightened sexual maturity, deliver high egg production rates annually, and generally have a short interval between generations [17]. This information was further supplemented by Bednarczyk et al., who described possibly being able to adopt semen dilution and artificial insemination to obtain a more significant number of offspring just from a single rooster [6]. Combined with the logistical perks mentioned above in the first paragraph, the chicken embryo becomes a fitting choice for studying transgenerational epigenetic research [17].

The primary terminology associated with this experimental model is the chicken Whole Embryo Culture (cWEC) technique, which can be defined as the culturing of the chicken embryo and the follow-up of its developmental process from stage X blastoderm formation to

hatching [51]. Early iterations of this technique can be dated back to the 1950s, whereby New's explantation of the blastoderm was first introduced by New, who successfully carried out in-vitro cultivation of chicken embryos [24]. Nevertheless, poor efficiency was reported, citing relatively low growth rates and only viable for early-stage development [24]. Subsequently, researchers sought more streamlined approaches for cWEC to advance chicken embryo research. This led to a consolidated effort to focus on utilizing and improving in-ovo designs for embryonic culture, as evident in several papers [52-54]. The in-ovo concept, usually noted as windowing, is etching a small window in the eggshell and covering the hole with a transparent seal to provide limited optical visibility. This was demonstrated by Speksnijder and Ivarie, who performed the procedure by carving a hole using a micropower tool and sealing the gap with plastic film and paraffin wax [22]. Subsequently, later iterations of this concept were conceived, one of which being the surrogate eggshell system. The surrogate model is an accessible alternative model that also employs the windowing technique, but the method is performed on another eggshell of a different avian species [20]. One fine example is the culturing of the quail embryo in a foster chicken eggshell, where Kamihara demonstrated the applicability and feasibility of the idea for morphological observations and embryonic manipulation [36]. Although windowed and surrogate models are usually utilized for similar research purposes, they vary in unique advantages and disadvantages, depending on the experimental requirements. Case in point, Borwornpinyo et al. observed that surrogate cultures had higher hatchability than windowed cultures, citing possible reasons for the size of the window and external factors [20]. Not long after, researchers also began exploring the idea of culturing the embryo in shell-less systems. Adopting a shell-less approach facilitates greater ease of access to the embryo and accommodates more advanced research capacity such as microsurgery applications [26, 53, 55]. This ex-ovo concept typically develops the embryo in synthetic models such as Petri dishes, plastic films, or artificial mediums [27]. When New

devised the embryonic explantation, he also pursued implementing the ex-ovo system by explanting the embryos into plastic containers and Petri dishes [24]. However, the ex-ovo models were poorly optimized, and it was only several years later, in 1974, that Auerbach et al. revised the culture method and managed to increase the viability for long-term embryonic cultivation [32]. Since then, numerous variations of the ex-ovo concept have emerged, each with its own set of unique attributes and utility. There is ongoing discussion regarding the preferred choice between incorporating in-ovo or ex-ovo models for research. Ex-ovo models usually maintain superiority in accessibility, optical visibility, and the capacity to accommodate more advanced research techniques [27].

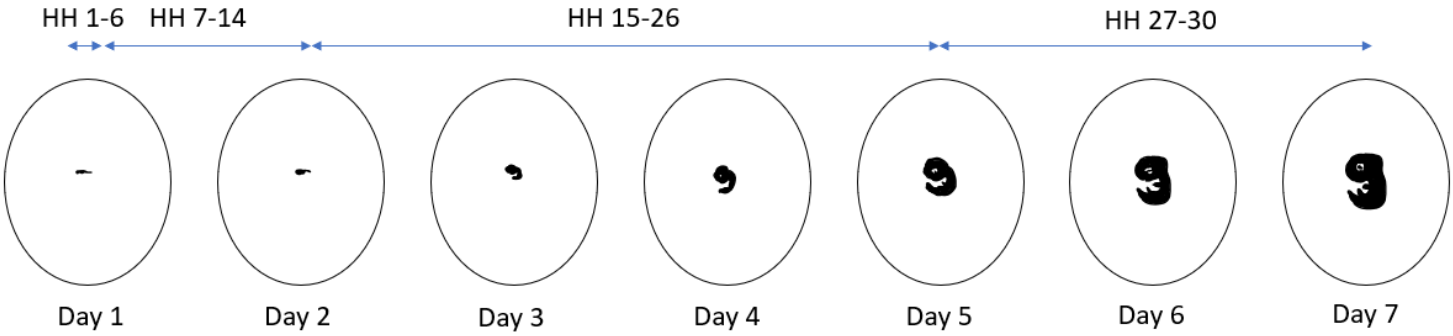
On the other hand, in-ovo models are uncomplicated, easy to perform and attempt to retain the natural environment of the chicken embryo [51]. Naturally, variations in both categories still suffer from prevalent complications such as contamination or inability to integrate egg turning, which is essential for embryonic survivability [27]. It all depends on the nature of the experiment itself, whether the goal is catered towards shorter-term studies or long-term studies with hatching capabilities.

Hamburger-Hamilton Stages

Named after its creators, Viktor Hamburger and Howard L. Hamilton, the Hamburger-Hamilton (HH) is an extensive compilation series of 46 chronological stages that span the entire developmental progress of a chicken embryo right from the laying of the egg to the ending of a newly hatched chick [48]. Its sole purpose is to illustrate the different embryonic stages in great detail, having the practicality of identifying, designating, and segregating developmental segments into ascending 'stages' based on morphological landmarks [48]. The HH model currently has high adoption in chick embryology due to its high accuracy in characterization for all embryonic stages, making it the preferred and standardized form of morphological indication [56]. There is considerable relevance in experimental embryology, especially when accounting for its role in in-ovo and ex-ovo chicken embryo research. Researchers can form valid comparisons when analyzing a normal chicken embryonic development and an artificially cultured one. This is evident in several papers which described any notable morphological observations or growth differences based on the HH stages benchmark [27, 57, 58]. One prime example would be identifying calcium deficiency deduction in shell-less culture systems based on biological comparison and observed abnormalities via HH analysis [43].

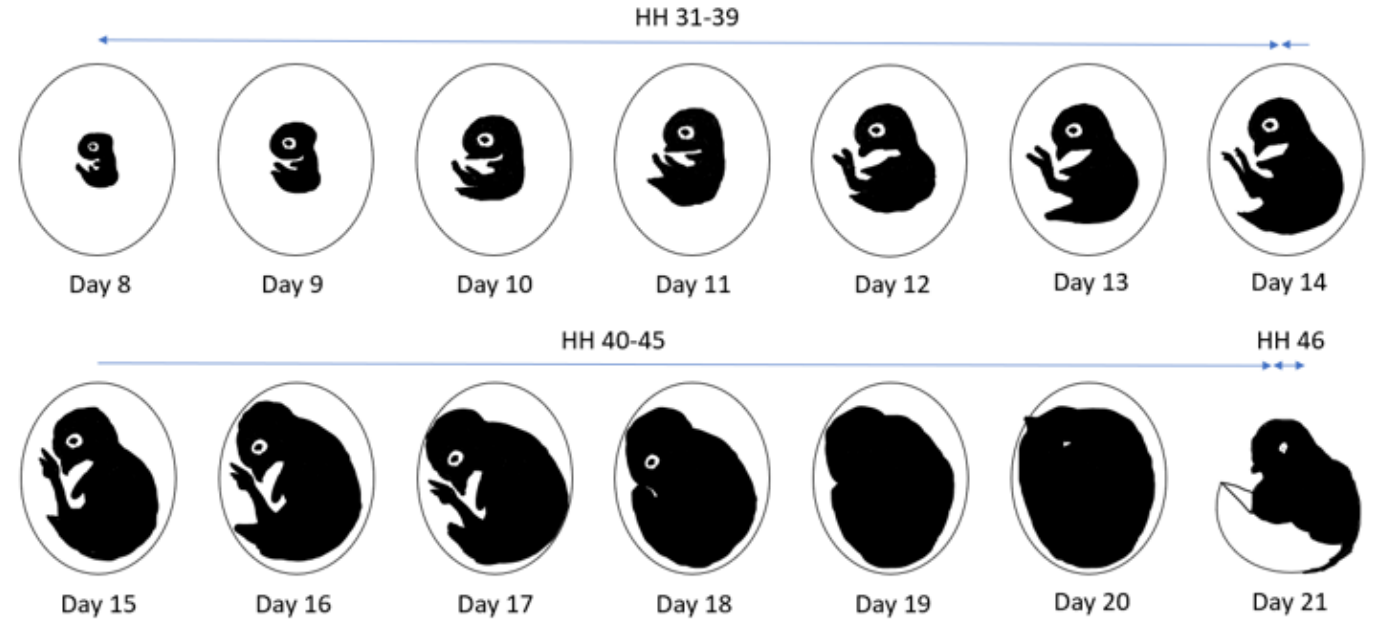
The HH stages can be loosely classified into several main divisions with subsegments, all of which are per distinct morphological landmarks as they have somewhat of a stereotypical appearance. These are the primitive streak formation, followed by somitogenesis, brachial arch evolution, limb development, and ends with feathers, eyelid, and beak formation for end-stage development [48]. Hamburger and Hamilton chose this characterization method as it was not reliant on the embryo age to gauge its development; doing so would introduce more variability as the initial growth is often challenging to pinpoint [59]. This is because internal fertilization first occurs within the mother hen, allowing for a brief development period [59]. When the egg is laid afterward, further embryonic development will be stalled until the incubator begins [59].

This meant that the eggs could have begun incubatory processes at different developmental stages, which would result in an inaccurate comparison if the embryos were to be analyzed based on maturity. Moreover, any disparity in incubation temperature and breed genetics is likely to influence developmental progress and timing. Below are illustrations depicting the chicken embryo developmental growth in relation to Hamburger-Hamilton Stages.



Incubation Period (Days/Hrs)	19-24 Hrs	24-48 Hrs	49-120 Hrs (2-5 days)	120-168 Hrs (5-7 days)
Hamburger & Hamilton (HH) Development Stages	HH Stages 1-6	HH Stages 7-14	HH Stages 15-26	HH Stages 27-30
Primary Landmarks	• Presence of primitive streak • Formation of head fold	• Growth of head fold formation • Cranial development	• Formation of limb and tail buds • Eye formation and pigmentation	• Continued growth of limbs and tail buds • Beak and toe outgrowth • Feather germination

***Figure 1**: Hamburger and Hamilton Stages of Development from Day 1 to Day 7*



Incubation Period (Days)	7-13 Days	14-20 Days	21 Days
Hamburger & Hamilton (HH) Development Stages	HH Stages 31-39	HH Stages 40-45	HH Stages 46
Primary Landmarks	• Progressive growth of feathers	• Maturity in size • Gains length and height	• Hatching of a new grown chick

***Figure 2**: Hamburger and Hamilton Stages of Development from Day 8 to Day 21 (Hatching)*

Primitive Streak Formation (HH 1-6)

This segment encompasses HH stages 1 to 6, where it primarily focuses on the formation of the primitive streak prior to incubation. During Stage 1 (Pre-Streak), the primitive streak has yet to be seen, but an 'embryonic shield' can already be observed [59]. This can be attributed to the accumulation of cells in the posterior section of the blastoderm [60]. Stage 2 (Initial Streak) marks the actual manifestation of the streak itself, about 6-7 hours into incubation [59]. In Stage 3 (Intermediate Streak), roughly about 12-13 hours into development, its appearance becomes more apparent [48]. Increased growth is noticeable in Stage 4 (Definitive Streak), with the streak acquiring its maximal length, and several characteristics can be seen; primitive groove, pit, and Hensen's node [48]. 19-22 hours later, the formation of the head begins to take place in Stage 5 (Head-Process), with Stage 6 (Head-Fold)

denominating the presence of the head-fold itself [48]. Hamburger and Hamilton also noted a transitory phase, concurrent somitogenesis and head-fold formation [48].

Somitogenesis (HH 7-14)

HH Stages 7 to 14 are dependent on the number of pairs of somites that can be confidently observed. This criterion is vital during this developmental phase from 23-53 hours, whereby the increasing pairs can be practical for assessment purposes [48]. There are two somites during Stage 7 (One Somite), but one of them is not yet well-defined to be included in the estimation [48]. In addition, several neural folds can be observed in the head-fold region [48]. By Stage 8 (Four Somites), the somite pairs have doubled, and blood islands can be found at the base of the blastoderm [59]. Optic vesicles and seven pairs of somites are detected in Stage 9 (Seven Somites) [59]. Signs of cranial development begin to show in Stage 10 (Ten Somites), whereby this is indicated by the presence of three brain vesicles and a hint of cranial flexure [48]. About 40-45 hours later, Stage 11 (Thirteen Somites) displays five distinct appearances of hindbrain neuromeres alongside the neuropore's closing process [48]. Stage 12 (Sixteen Somites) marks the closure of the neuropore and the establishment of the primary optic vesicles and stalk [48]. Finally, the cranial progress is well developed by Stage 13 (Nineteen Somites). The telencephalon is enlarged, and the headfold region encapsulates the midbrain, forebrain, and anterior section of the hindbrain [59]. Stage 14 (Twenty-Two Somites) marks the final process of this segment, with evidence of branchial arches formation and invagination of the optical vesicle commences [48]. Beyond this stage, characterization of the somite pairs becomes increasingly arduous due to the dispersion of the anterior most somites in the mesoderm [59].

Branchial Arch and Limb Formation (HH 15-26)

From HH 15 onwards, the characterization method extends to externally visible structures such as limb buds, visceral arches, or other morphological landmarks [48]. Stage 15 (50-55h) comprises amnion encapsulation over fourteen somites and the complete formation of the optic cup, aperture, and the third branchial arch [59]. The wing and tailbud can then be observed in Stage 16 (51-56h) with amnion extension, including eighteen somites [59]. At Stage 17 (52-64h), the limb buds are prominent, formation of nasal pit begins, and amnion extension continues coverage of up to twenty-eight to thirty-six somites [59]. Production of allantois initiates in Stage 18 (65-69h), with the amnion fully closing and enlargement of limb buds occurring; leg buds appearing more considerable than the wing buds [59]. Roughly three days into incubation, somites begin extending towards the tail in Stage 19 (68-72h), and the tailbud shows curvature towards the head [59]. The allantois becomes vesicular, with signs of pigmentation in the eye on Stage 20 (70-72h) [48]. Stage 21 (3.5d) is where certain hallmarks are more noticeable; the limb buds are more enlarged than the wing buds, and the allantois extends to the head region [59]. This extends to Stage 22 (3.5d) and Stage 23 (3.5-4d), with the limb and wing buds growing in length and width, and distinct eye pigmentation can be seen [59]. On Stage 24 (4d), the limb and wing buds have reached optimal size development, followed by the initiation of toe plate formation [59]. Limb definition continues in Stage 25 (4.5d), with the elbows and knees of the embryo begin surfacing [59]. Stage 26 (4.5-5d) marks the end of the significant limb development, with the limbs considerably lengthened and demarcated with the first three toes present [48]. Beyond this stage, the embryo will be characterized by beak, toes, and feather definition alongside continued limb growth progression.

Late-Stage Development (HH 27-46)

Five days into the incubation on Stage 27 (5d), the early beak structure is barely discernible, and groove marks are indicated between the toe digits [59]. Beak outgrowth is evident on Stage 28 (5.5d), and the toes can also be observed on the chick, with four out of five visible [59]. Stage 29 (6 d) is where a couple of anatomical changes occur. First, bending of the wings at the elbow and specialization of the limb appears, identified by the webbing of the toes [59]. Next, stage 30 (6.5d) denotes the arrival of the egg tooth, with the beak being more pronounced, and the limb segments are well-defined at this point [59]. Feather germination also begins initiation at this stage, although not entirely throughout the whole body [59]. From this point onwards, development in the later phases can span a few days. For example, Stages 31 to 39 (7-13d) encompass the progressive growth of the feathers, with each day getting more conspicuous and prominent [59]. Throughout Stages 40 to 45 (14-20d), the chick matures in size, gaining length and height in the toes and beak [59]. Finally, on Stage 46 (20-21d), a newly grown chick is hatched.

Role of Egg Turning during Incubation

In artificial incubation, one of the essential factors vital for the optimal growth and development of the chicken embryo is the turning or rocking of the eggs. The turning of eggs during the incubation stage is a natural behavior among avian birds, and it contributes significantly to both the physical and physiological aspects of the embryo [61]. These include the formation and development of the extraembryonic membrane, embryonic growth, and the usage of albumen [34, 61, 62]. A recent paper explained in their review that embryonic development, ventilation, and egg positioning are all attributed to the effects of egg turning. These, in turn, can influence downstream processes such as heat transfer, gaseous exchange, nutrient accessibility, internal water loss, and adhesion of the extraembryonic membrane to the embryo [63]. Failure to adhere to this requirement of good turning frequency is suggested to impose a detrimental effect upon the embryonic growth rate, development, and nutrient uptake. This is supported by multiple studies that reported decreased absorption rate and retarded growth in the chicken embryos [35, 64, 65]. Other reported observations include premature shell adhesion to the extra-embryonic membrane and subsequent abnormal developments following this premature adhesion [65-67]. One correlation between increased mortality and premature adhesion is proposed to be the restricted growth and development of the chorioallantoic membrane [67-69]. This is also supported by other findings that the chorioallantoic membrane was observed to have stunted growth with the absence of proper turning, resulting in a reduced capability to spread around the inner surface of the shell membrane [70, 71]. This can lead to inefficient absorption of the albumen [64] and poor respiratory gas exchange [71, 72]. Therefore, adequate egg turning remains a crucial component in the viability and success of artificial chicken embryo culture systems.

Elements constituent to the specifics of egg turning remain to be discussed, namely the timing, frequency, and angle. These factors contribute to the effectiveness of the egg turning

methodology, and past research has extensively documented the optimal parameters to increase the hatchability rate. Regarding the element of timing, several papers have identified and come to a consensus that a 'critical period' exists regarding the application of egg turning [35, 62, 65, 69]. However, these findings differed in the associated duration and the number of days. For example, an early study by Kaltofen suggested that the critical period lies within the second week of incubation (day 7 to 14) [73]. Another paper also highlighted that days 7 to 14 were vital for egg turning [8].

Conversely, other researchers reported that egg turning in the first week of incubation is more significant for embryonic development [8, 35, 68, 69]. Elibol and Brake noted that the percentage of mortality and malposition of the chicken embryo increases during the absence of turning in the first week, especially during days 0 to 2, where the detrimental effects are the most pronounced [69]. Likewise, supplementary reports also mentioned that this turning mechanism is particularly crucial during the first week of incubation under the long distance between the embryo, albumen, and shell, aiding in the facilitation of gaseous exchange and nutrient absorption [63, 74]. The pointers above help to reiterate the notion that sufficient adequacy and 'optimal window' of egg turning application directly impact the success of avian embryonic development.

The second and third elements to be reviewed are the influence of turning frequency and its rotation angle. In their natural incubation state, Landauer observed that hens maintained an approximate turning frequency of 96 times daily [75]. To determine if the turning rate influenced embryonic growth, Kaltofen and Ubbels experimented and discovered that when the eggs turned at least 24 times a day, they displayed higher hatchability results than those not turned as often [63]. This assessment was further validated years later when Wilson demonstrated that the high turning frequency of 96 times a day enhanced in-ovo development and hatchability rate [8]. Elibol and Brake also presented similar results when comparing

turning frequencies of 24, 48, and 96 times a day, with 96 times exhibiting the characteristics of an optimal turning frequency [76]. However, in the real-world setting, it is impractical for commercial incubators to run at such a high setting as this raises equipment maintenance fees [63]. Coupled with the findings by Freeman and Vince that the additional benefits gained between 24 and 96 times are marginally small, it would stand to reason that turning 24 times daily in an hourly fashion is more than sufficient to provide stated beneficial effects [67]. This leaves the subject of the optimal tilt angle required to complement and augment hatchability results within the limits of operating capabilities. In the 1990s, researchers reported that natural embryonic development was reproduced when eggs were artificially rotated 90 degrees hourly. They also determined that 90 degrees is the optimal setting and was achieved by turning the eggs 45 degrees side to side [77, 78]. Subsequently, this was adopted for typical industrial practices as it also satisfied the operational conditions of a commercial incubator [62, 79, 80]. To better understand the distinct role of the tilt angle, further analyses were carried out to ascertain if turning at a reduced angle could induce any detrimental response to the embryo's development. An experiment in 2006 highlighted their findings when the chicken eggs placed in a vertical setting were subjected to different turning angles of 35, 40, and 45 degrees, at a rate of 24 times rotating frequency daily. It was revealed that the percentage of malpositioned embryos was elevated by 35 degrees compared to the 40 and 45-degree angles [79]. Similarly, when comparing eggs turned at different angles, another research article reported higher embryonic mortality and decreased hatchability when the eggs were turned at 15 degrees compared to 45 degrees [62]—as such, maintaining a 45 degrees angle with the necessary concomitant hourly turning frequency is recommended for industrialized or research incubation purposes.

In-Ovo Culture System

The derivation of in-ovo approaches for chicken embryos can first be observed when there is a need for biological, developmental studies, and manipulation in avian bird species. During its infancy stages, in-ovo-related techniques were only applicable for the first few days of embryonic development, from the blastoderm formation to the embryo's growth [51]. Subsequently, researchers found out that these techniques can be expanded to include later developmental stages in the chicken embryo, resulting in an emergence of new research interests, including transgenicity [21], angiogenesis [13], and toxicology [13], and biomedical studies [27]. In general, the in-ovo method can be described as creating a window in the eggshell to gain access to the embryo during the culturing process and retain its natural environment as closely as possible [23]. This method has several utilities to exploit depending on the experimental goals and project direction. For instance, sealing the eggshell window with transparent material can facilitate time-lapse imaging and developmental observation [81]. In addition, accessibility to the embryo via this windowing process significantly enhances the progression of transgenic avian production by aiding in increased capacity for viral infection delivery [82].

The in-ovo culture system can be broadly classified into the windowed and the surrogate egg model. As aforementioned before, the windowing approach has fundamental roots and is demarcated to be the primary concept pertaining to the idea of in-ovo avian culture. On the other hand, the surrogate technique stems from a variation of the original concept. Instead of hosting the embryo in the native eggshell, it is transferred to another avian eggshell for culturing [20]. One could also say that the surrogate method improves the original model, bringing about several perks such as larger window space for embryonic manipulations and an increased survivability rate overall [83]. However, that does not mean that the windowed model wholly pales compared to the surrogate model; barring the need for extra workspace and

accessibility, the windowed egg is more time and cost-efficient, considering that additional avian eggs need to be acquired beforehand for the transfer.

Currently, both models are heavily utilized in transgenic chicken technology and the production of recombinant proteins in eggs [21, 47]. This is evident in the early attempts to develop novel transgenic birds with retroviral infection [21, 82]. Latest developments have also included the adoption of in-ovo electroporation [84], which greatly enhanced the process of producing modified competent primordial germ cells and paved a new avenue in genome-edited chicken embryos [85]. Production of germline chimeras can be instrumental in certain circumstances, mainly when applied to propagate endangered bird species. For example, Li et al. successfully demonstrated the feasibility of producing duck-chicken germline chimeras by transferring stage X duck blastodermal cells to chicken embryos [86].

It is crucial to align scientific interest and experimental goals to select the appropriate chicken embryo model for in-ovo research. Unlike the distinction between ex-ovo culture systems, the in-ovo models are somewhat similar in design and methodology processes, excluding that they usually cater to specific purposes. Table 1 below summarizes the necessary information describing the use cases, strengths, and weaknesses of the in-ovo models. Further clarification on their characteristics will also be provided in the ensuing sub-chapters.

Table 1: Summary of the different in-ovo chicken embryo models

Culture Model	Setup Description	Applications	Pros/Cons	References
Windowed	Native eggshell with a small window for accessibility. Windows can be covered with opaque or transparent material for optional optical visibility.	Time-lapse imaging Developmental studies Transgenic avian production	Advantages: - Cheaper - Suitable for early/mid-stage embryonic experiments - Quickest setup - Calcium supplementation from eggshell to bolster growth Disadvantages: - Lower hatchability and survivability than surrogate model - Limited accessibility and workspace due to smaller window - Requires window sealing optimization to prevent contamination	Kulesa et al. [81] Cuperus et.al [87] Lee et.al [88]
Surrogate	Eggshell from another avian species with a window-like piece cut out for accessibility. Windows can be covered with opaque or transparent material for optional optical visibility.	Time-lapse imaging Transgenic avian production Recombinant protein production Embryonic manipulation	Advantages: - Cheap - Suitable for all-stage embryonic experiments - Calcium supplementation from eggshell to bolster growth - Increased workspace due to larger window size Disadvantages: - More time consuming than windowed due to additional steps required - Requires window sealing optimization to prevent contamination	Borwornpinyo et.al [20] Mozdniak [16] Liu et.al [86] Speksnijder et.al [22] Naito et.al [47]

Windowed Eggshell Model

The windowing technique was first introduced in the early 2000s when it became a method of choice for genetic modification via the transduction of the chicken embryo with retroviral vectors [82, 89]. The general procedure typically involves using a micropower tool to cut a small hole in the eggshell, allowing researchers to gain access to the blastoderm and deliver the viral vector payload with greater precision, hence improving the efficiency of avian transgenic experiments in terms of labor intensity [22]. Generally, after the injection is completed, the window will be covered with a piece of shell membrane and sealed with cement, reducing the chances of external contamination [22]. Realizing its potential for adaptation, other scientists began to inculcate this method into their developmental experiments for various specific purposes. As a result, the windowing technique granted access to the blastoderm in its early stages. Furthermore, it generated new opportunities for other biological and developmental analysis types, such as chorioallantoic membrane studies [90] and even tumor kinetic studies [91]. In addition, real-time observation of the embryo can also be facilitated by substituting the opaque shell membrane used to cover the window with a transparent material.

This method of employment can be observed in several literature papers, namely in the application areas of time-lapse imaging [10, 81], developmental studies [27, 54], and transgenic production of germline chimera [16, 22]. However, the foremost notable advantage of the windowed model is its capacity to retain the natural habitat of the chicken embryo as closely as possible, without the need for the embryo to be developed outside of its shell. This is significant because eggshell plays a critical role in embryonic development; calcium supplementation provides about 80% of the total requirement [92]. Furthermore, without the primary source of calcium, the embryo will become hypocalcemic over time, which will drastically affect bone mineralization and lead to overall growth retardation [92]. Despite this, windowing remains the most basic form of in-ovo technique because although retention of the

natural environment is a plus, the size of the window is often too small to accommodate proper optical accessibility and facilitate more advanced laboratory techniques [16].

Different papers described varying rates of hatchability and survivability, depending on the experimental parameters and method variations implemented. For instance, one article observed an 8.2% hatchability rate for eggs with standard windowing procedures [22], whereas another paper reported a 32% hatchability rate using a simple modification [21]. The variability may be attributed to the artificial introduction of air bubbles during the procedure, which can be detrimental to the embryo and decrease survivability rates [21]. According to several experts, the perturbation that occurs during windowing results in an additional increase in mechanical tension, which weakens the integrity of the embryonic structure [93, 94]. Furthermore, the newly introduced airspace also encourages localized dehydration and promotes friction between the blastoderm and surrounding membranes [93]. In light of this matter, several solutions have been proposed and implemented to reduce air bubbles occurrence and reduce embryonic mortality. For example, Speksnijder and Ivarie suggested using phosphate-buffered saline (PBS) solution to coat the underlying shell membrane during the windowing process, such that when the membrane is removed to facilitate access to the blastoderm, PBS and not air, would be drawn into the egg, therefore limiting air influx [22]. Andacht et al. also devised an enhanced approach to sealing the eggs using hot glue, which aids in expediting trapped air removal by enabling passage across the glue during cooling [21]. All in all, these implementations can help improve the viability of the windowed model and will allow it to retain relevancy in transgenic avian production and developmental studies.

Surrogate Eggshell Model

The surrogate eggshell culture method is regarded as one of the extensions of Perry's concept for a complete system of the chicken embryo, which is a modification of the windowed eggshell technique [51]. When the embryo reaches early embryogenesis, it is transferred to another recipient eggshell which is generally more significant for further incubatory development till hatching [51]. Once the transfer is complete, the recipient eggshell will be sealed, typically with a plastic film, and subjected to standard incubation conditions. This process can be derived from having the primary need to utilize a larger window size for enhanced optical visibility and accessibility for embryonic manipulation and increase the hatchability rate based on existing methods [19, 36, 51]. However, researchers could only attain a 7% hatchability rate in the early stages when this technique was in its infancy [51]. Subsequently, newer studies began to focus on optimizing this aspect, with several bands of scientists managing to achieve higher hatchability rates in surrogate eggshell systems. In one particular case, a research group led by Naito raised the hatchability rate to 34% simply by replacing the thick albumen with thin albumen at the blastoderm stage [95]. Another study led by Kamihira also experimented with external calcium supplementation, which resulted in an overall increase in survivability and hatchability rates [36]. The increased rates meant that researchers could employ this method with a higher confidence level to conduct experiments that require longer-term survivability or hatching of the chicken embryos.

Once the hatchability rates had been ascertained to reach satisfactory levels, other novel ideas revolving around the surrogate egg system began to arise. For example, Liu et al. investigated the feasibility of implementing a variety of eggshells and albumens from different avian species and obtained a wide range of hatchability percentage rates from 7.7% to 60%, depending on the interspecific egg white used [83]. However, these rates have dependent on the phylogenetic distance of the donor and recipient species components [83]. In addition, other

researchers explored alternatives in optimizing the window size of the surrogate eggshell and perfecting the sealing of the window after embryonic transfer [20, 21]. All these measures aim to increase the throughput of this proxy system and find an increasing use case for developmental biology.

The surrogate system can be considered a variation of the windowed eggshell, with both models having a window for accessibility and the embryo being cultured in an actual eggshell [21]. It differs because the eggshell utilized for culturing usually belongs to another avian species, whereas the windowed eggshell is intimate to the chicken embryo itself [33]. With a current need for a larger workspace for avian species with smaller egg sizes, such as quails, this model allows greater accessibility to the embryo for manipulative studies and general observation [36]. Another perk of the surrogate system can be attributed to incorporating the natural eggshell as a culture medium. A well-established fact regarding developmental growth is that the embryo initially derives most of its calcium source from the egg yolk and then from the eggshell in later stages [40, 43, 96]. The surrogate model has an added advantage over ex-ovo models in that the embryo can derive its additional calcium needs via the eggshell, which is crucial to its sustainability and growth [41].

One should also note the limitations of adopting this model when considering the nature of the experiment itself, especially for the applications of chimera research. As aforementioned, there is an ongoing discussion concerning the correlation between the phylogenetic distance of the host and recipient avian species and the hatchability rate of the recipient embryo [83]. In the article, Liu summarized that several factors might reduce hatchability rates, requiring further investigation to supplement the results [83]. Another aspect to note is the sealing component for the eggshell, which is mandatory to cover the gap and prevent bacterial infiltration. Naturally, the material used to seal the gap must be transparent to facilitate optical visibility for observation purposes. In this instance, plastic wraps are often applied alongside a

rubber band to secure the film after sealing. The type of plastic wrap used also plays a role in the contribution to embryonic survivability; Borwornpinyo et al. discovered that eggs sealed with Saran wrap compared to Handi wrap had higher mortality rates between days 5 to 8 [20]. One possibility can be attributed to the permeability properties of the plastic film, which can influence gaseous transfer and moisture levels within the egg [20]. That being said, this model offers slightly more minor reproducibility due to the effort required to construct a completed surrogate system and not being able to reuse the host eggshell for future uses. Not being autoclavable also increases the risk of cross-contamination when transferring the donor embryo to the recipient eggshell, even in a sterile environment. Despite these limited downsides, surrogate eggshell models remain viable in developmental biology, especially in transgenic avian production [21].

Ex-Ovo Culture System

The need for a comprehensive, continuous observation and detailed analysis of embryonic development has led to the rise of shell-less culture systems (ex-ovo) in place of other traditional culture systems [57]. Compared to avian embryo in-ovo models, existing solutions such as windowed or surrogate eggshells techniques are often laborious and can be challenging to visualize embryonic activity due to limited optical windows [32]. In addition, these methods can induce unwanted side effects, such as chorioallantoic membrane inflammation, in the case of windowed eggshell systems [97]. Conversely, explantation, which is the transfer of the specimen to an in-vitro system such as a culture medium, resolves several of the issues previously indicated [32]. Initially, the explanted embryo is often cultured using the petri dish model and is adequate for early-stage developmental studies and manipulation. However, it is inept for long-term adaptation and faces shortcomings such as the required time and skill level [24]. Over time, scientists develop new strategies and devise new methodologies, such as conceiving a weigh boat vessel setup of different materials. These include inorganic materials such as plastic film [57] or biocompatible and gas permeable components like polydimethylsiloxane [98]. Newer systems incorporating polydimethylsiloxane are more robust and feature more improvements, such as improved optical visibility and accessibility [27], whereas more straightforward, revised methods like the plastic film vessel thrive in its candor and economic edge for shorter-term studies [25].

Similarly, with the in-ovo culture systems, the ex-ovo models can deliver a wide range of biomedical applications and developmental research. The prevailing ones include imaging modalities like time-lapsing [33], fluorescence [99], stereomicroscopy [33], and angiogenesis analysis such as biocompatibility studies [100] and drug kinetics [99]. Other alternative use cases cover microsurgery [26], tumor inoculation [91, 101], embryonic manipulation [100], and grafting [100]. These examples enabled the chicken embryo model to be extremely useful

and popular in molecular biology and cancer research. The caveat notwithstanding is that each model variation offers distinct benefits and drawbacks. Hence, it should be relevant that the appropriate design must be chosen with discretion and according to experimental goals. Table 2 below summarizes the currently accepted models in today's research practices and adoption. Subsequently, additional information will be provided in later sub-chapters, describing each model's characteristics.

Table 2: Summary of the different ex-ovo chicken embryo models

Culture Model	Setup Description	Applications	Pros/Cons	References
Petri Dish	Petri dish with optional lid and water bath for humidity control	Chorioallantoic membrane related studies - angiogenesis and vascularization Tumorigenesis Intravitreal injection	Advantages: - Cheapest - Suitable for early-stage embryonic experiments - Quickest setup Disadvantages: - Low viability - Non-turning capacity - Unnatural culture environment	Wissler [102] Kistler et.al [103] Oppitz et.al [101] Uchibayashi et.al [91] El-Ghali et.al [24]
Plastic Film (polyethylene, polyvinylidene chloride, polymethylpentene, etc.)	Polymer film attached to a cup and shaped like a 'hammock,' tied down with elastics and covered with an additional lid or layer of film	Time-lapse imaging Microsurgery Chorioallantoic membrane related studies - angiogenesis and vascularization Embryonic development observation	Advantages: - Cheaper - Capable of early/late-stage embryonic experiments - Quicker setup - Portable - Decent viability Disadvantages: - Potential loss of vessel integrity due to elasticity of plastic film - Non-turning capacity - Dependent on type of polymer material used - Minimal reusability after each use	Dunn [57] Dunn et.al [97] Yalcin et.al [26] Kamihara et.al [36] Scadding & Steven R [104]
Polydimethylsiloxane	Cubic PDMS construct with an internal oval-shaped curvature and covered by a plastic film Cubic PDMS construct with an internal cuboid structure made up of thin, polycarbonate assemblies Biomimetic PDMS model mimicking the elliptical geometry of an egg, with a PDMS cap securing the top	Time-lapse imaging Embryonic manipulation Chorioallantoic membrane related studies - angiogenesis and vascularization Embryonic development observation Biocompatibility studies	Advantages: - Robust and portable - Suitable for early and late-stage embryonic experiments - Quick setup - Good viability - High reusability and can be sterilized - Turning capacity (dependent on model variation) Disadvantages: - Longer production period (1 day) - Thickness of material affects oxygen permeability and viability of embryo - Ill-suited for short-term, high throughput experiments	Lai & Liu [9] Huang et.al [105] Ishak et.al [27]

Petri Dish Culture Model

In the past, explanting had always been a favorable option for studying the early-stage development of the chicken embryo. One particular alternative is the New technique, which allowed researchers to culture the embryo in-vitro by separating it from the yolk and saving as much vitelline membrane as possible [24]. Despite its breakthrough, this approach has multiple shortcomings, such as intrinsic difficulty, time consumption, and required skill level. This makes it unsuitable for time-sensitive applications and long-term cultivation studies [24, 26, 32]. In addition, new and improved procedures were required to facilitate the need for rapid observation of a large number of chicken embryos and expedite visual examinations through transmitted light. As a result, numerous researchers have adopted the 'Petri dish' culture method, whereby the embryo is directly transferred to a petri dish as the medium with or without additional supplementation [24, 32, 90]. The Petri dishes can then be placed in a water bath for humidity control or in incubators with the appropriate environmental conditions. This enabled rapid deployment of sample groups while relatively maintaining an inexpensive cost with reduced time consumption [24]. This method is straightforward, cheap, easy to reproduce, and time-efficient, especially for various studies on early-stage embryo maturity.

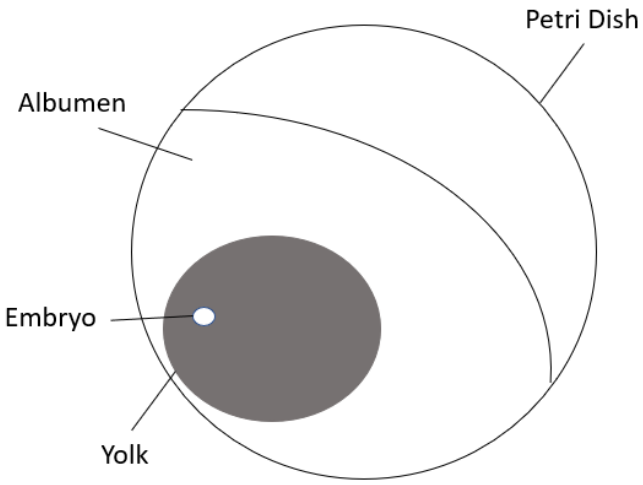


***Figure 3**: Schematic of a basic petri dish culture setup*

Despite its simplicity, this ex-ovo technique does have its fair share of drawbacks. One distinct aspect is the low viability rate for the chicken embryo. El-Ghali et al. reported that they could only obtain a two-day maximum survival window with a 50% average mortality rate [24]. Another study also delivered similar results, with the embryos having a 50% mortality rate during the first three days post-incubation [32]. It also described other challenges, such as small undetected leaks in the yolk membrane or malposition of the chicken embryo [32]. Although the authors were unable to derive an explanation, these outcomes can plausibly be attributed to the unnatural cultural environment; the flat surface of the petri dish is not an ideal platform to act as a receptacle and might disrupt the native biological processes that take place in an egg, which is oval. Furthermore, this experimental design limits the capacity of egg-turning, which is an essential component in sustaining embryonic survivability and development [34, 61, 62]. Nevertheless, this technique remains a viable option for early-stage embryonic manipulation studies if there is no need for long-term cultivation strategies.

Plastic Film Membrane Vessel Model

With the immersion of different culture systems implemented to cultivate chicken embryonic development, it is no surprise that researchers are constantly finding new cost-effective and reproducible methods to enhance their experiments. In order to employ more advanced laboratory techniques to closely monitor the microenvironmental and morphogenetic factors of the chicken embryo, a more straightforward and portable system is required [26]. Using plastic film as a foundation for the vessel was recently proposed by Japanese students in 2014, whereby a shell-less culture system to hatch chicken embryos using mainly plastic wrap was successfully devised [25]. Other papers have also reported adopting this methodology and modifying it based on their experimental needs and research [26, 57, 104, 106]. The basic setup typically involves a few items: a single piece of plastic film to form the culture chamber and

the lid, cup, or container acting as a holder, and an elastic ring to hold the plastic film in place [25, 26, 104, 106].

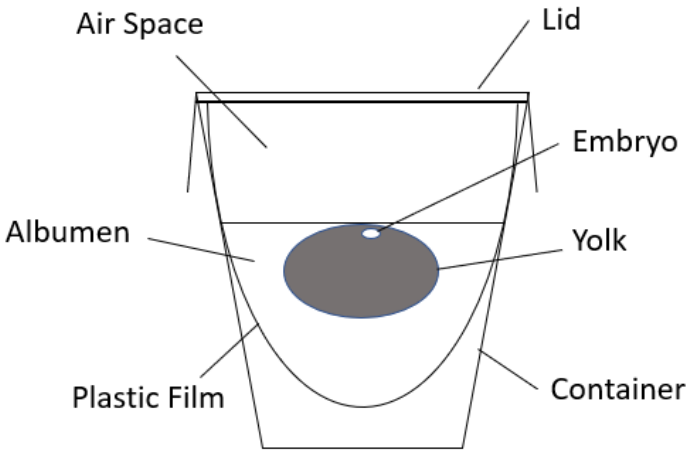


***Figure 4**: Schematic of a basic plastic film vessel setup*

Setups may also contain adaptations such as filter holes for ventilation or supplementations such as an antimicrobial solution or calcium supplementation [25, 43, 106]. Based on this technique, several papers noted varying results in the hatchability and survival rates of the avian embryo, depending on the experimental modifications. For example, Scadding observed that the survivability rate during the first week averages about 50% before dwindling to 15% over the second week [104]. On the other hand, Tahara et al. achieved over a 50% hatchability ratio with oxygen aeration and calcium supplementation [25]. The stark difference between the two reports highlights that the viability of the plastic film vessel depends on other experimental parameters.

The plastic film approach has its advantages and disadvantages, alongside specific challenges to overcome. Vessel integrity becomes an issue when an elastic material such as the plastic film is utilized. Adequate surface tension must be maintained when assembling, as insufficient stretching of the film develops weakness in the vessel chamber; hence it would not provide a stable foundation for the chicken embryo to develop upon. This was reiterated by an

article stating the importance of eliminating wrinkles in the film, leading to lower survivability of the chicken embryo [25]. The type of plastic film also comes into question when discussing the topic of permeability. Different materials such as polyethylene, polyvinylidene chloride, and polymethylpentene have differing oxygen and water vapor permeability properties, affecting the gaseous exchange functionality of the culture vessel [25, 107]. Therefore, researchers must consider their experimental needs before choosing the appropriate material for the setup assembly.

Furthermore, the basic plastic membrane vessel setup does not support turning functionality. The influence of turning on embryonic development has been extensively documented over the past decade and can be a critical component in developing whole embryo culture systems [8, 34, 35, 64, 68, 71]. The plastic film material is elastic and malleable, which means that the structural integrity will be compromised if the egg's contents are allowed to move.

Although the membrane vessel has minimal reusability after each use, its high availability, portability, reproducibility, and cost-effectiveness offset this. More accessibility is also offered for live surgical and manipulation applications due to having a bigger window size than other culture systems, such as windowed eggshells [26]. In terms of sterilization, it is not feasible to consider autoclaving due to the nature of the setup, and therefore, other disinfection methods must be examined, such as utilizing ultraviolet rays or simply with 70% ethanol. Nevertheless, this technique remains viable and relevant in modern animal research models.

Polydimethylsiloxane Vessel Model

The progression and continued pursuit of a more advanced, feasible solution for a robust ex-ovo culture system is driven by the need to overcome present limitations. These include poor visibility, low viability, and restricted use of mature laboratory techniques due to

small workspaces [9, 26, 36]. In addition, modern novelty approaches have led scientists to the idea of utilizing polydimethylsiloxane (PDMS) as a biomaterial in the fabrication process of the artificial ex-ovo culture system. PDMS is a silicon-based organic material and is well known for its versatility, consisting of microfluidics, medicine, cosmetics, and food [108, 109]. Moreover, it is vastly popular for a few reasons; it is chemically inert, possesses decent gas permeability, has good thermal stability, and provides good optical transparency [108, 110]. On top of that, its biocompatibility [111], non-toxicity, and economic availability also make it a widely accepted choice for biomedical applications and assemblies in biological studies [109, 112].

This approach in ex-ovo model research recently surfaced in 2015, whereby a study by Lai and Liu described the process of fabricating whole PDMS eggshells to culture avian embryos for in-vivo 3D fluorescent imaging [9]. It was achieved by placing a hard-boiled egg in the center of a box, filling up the remaining area with PDMS, and removing the egg afterward once the PDMS has cured [9]. This creates a negative space within the box itself in the shape of an egg and thus, enables the cultivation of the embryo. A separate study also adopted a similar methodology, except that the internal shape of the PDMS eggshell was cubic compared to the previous paper above [105]. Both designs delivered similar observations, noting that the chicken embryos could survive during the first week with high viability (80%) and faring better than traditional culture systems [9, 105]. In addition, Lai and Liu also remarked that the cultured embryos could reach hatchability using the PDMS vessel [9]. These constructs, however, did not faithfully replicate the morphological requirements in terms of the actual eggshell shape itself. A newer research article published in 2020 addressed the issue; developing a biomimetic transparent PDMS eggshell that mirrors an egg's elliptical geometry [27]. The fabrication process was shaped by a mold and spun inside a bioreactor to ensure even PDMS distribution [27]. The biomimetic eggshell had similar volumetric dimensions compared

to the chicken egg, and the authors revealed positive findings in the experiment; good viability within the first week of incubation and survivability up to the day before hatching [27].

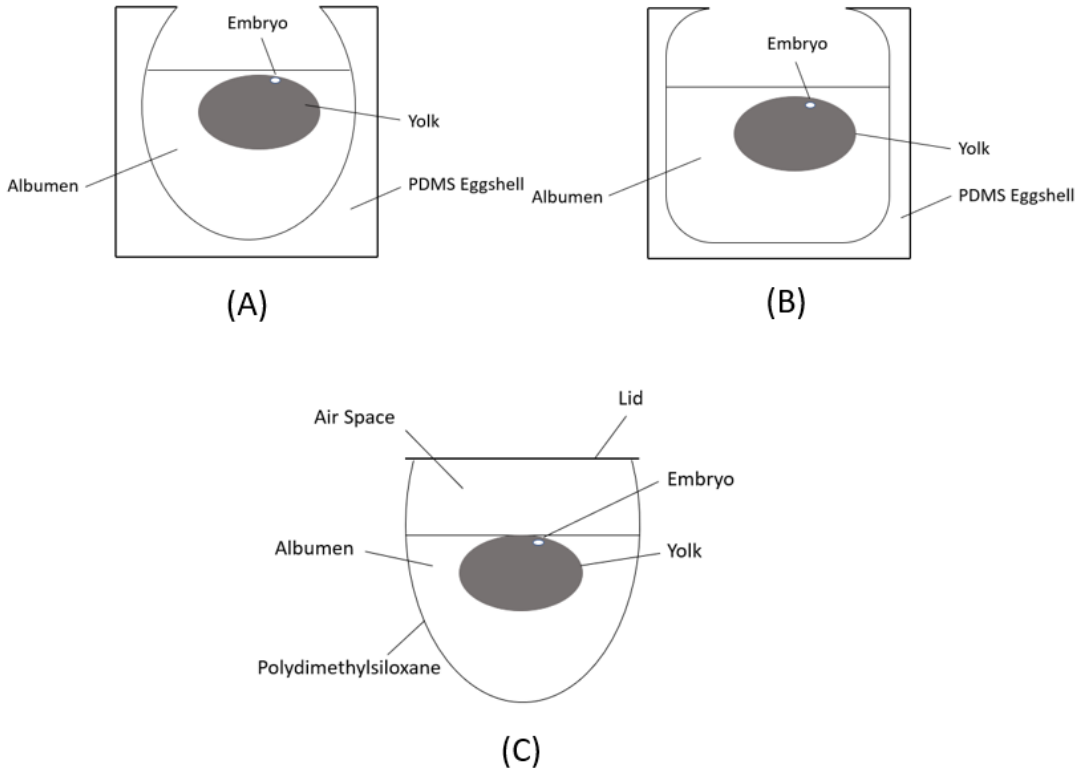


***Figure 5**: Schematic of the PDMS ex-ovo model designs: A) Egg-in-Cube PDMS vessel setup B) Variant Egg-in-Cube PDMS vessel setup C) Basic biomimetic PDMS vessel setup*

The gaps in existing PDMS fabrication technologies can be highlighted in the reports and designs of the PDMS culture vessels. Both variants in the egg-in-cube models, as compared to the biomimetic model, possess more inefficient manufacturing techniques in terms of consistency, the time required, and reproducibility [27]. On the other hand, the newer biomimetic model can be produced with general consistency in dimension and thickness, enabling using a bioreactor in rotation molding [27]. In addition, the cubic variants do not account for the phenomenon of egg turning, which is a naturally occurring process and plays a vital role in the biological development of the embryo [34, 61, 69]. Oxygen permeability is another crucial factor for consideration when adopting the cubic model approach because larger PDMS volumes are required than the biomimetic model. Since oxygen permeability directly

correlates with the thickness of the PDMS membrane [110], it would suffice to say that the membrane thickness would affect the viability of the chicken embryo. This is supported by Huang et al., who assessed the effects of different membrane thicknesses on embryonic survivability and demonstrated that increasing thickness from 0.1 up to 0.7 mm induced higher mortality during the first week of incubation [105].

Utilizing the PDMS vessel system does bring certain assets to the table. The most considerable distinction is its all-around optical transparency, which proves valuable when dealing with embryonic manipulation and imaging platform applications such as fluorescent imaging and microscopy. Several research papers above have taken advantage of this attribute with great success [9, 27, 105]. Moreover, the surface of the PDMS maintains multiple valuable properties, such as high inertness, low surface energy, and strong hydrophobicity [110]. The low surface energy and inertness discourage other materials from being deposited into the PDMS [110], and the hydrophobicity reduces bacterial attachment or penetration [113]. These innate attributes make it ideal for PDMS as a biomaterial to culture avian embryos. Last but not least, despite having a 24-hour curing process to fabricate one PDMS eggshell [9, 27], it is ameliorated by its reusability and capacity to be sterilized, making it superior to other ex-ovo culture systems when it comes to long-term cultivation functionality and reproducibility.

The transparent PDMS vessel does have its fair share of drawbacks amidst its advantages. Although the PDMS culture vessel has excellent optical transparency, it will accumulate impurities and particles on the surface over time, which would obscure and diminish the clarity of the vessel. Thus, even if the vessel can be recycled for embryo cultivation, it may not be viable for optical modalities for sideways imaging unless viewed from the top. Furthermore, the manufacturing process can be time-consuming and laborious [27], which means this methodology is unsuitable for experiments requiring rapid output and fast reproducibility. If the PDMS vessels are prepared beforehand, the issue will be resolved.

It is also essential to remember that PDMS adoption is different from an actual eggshell. A couple of sources have pointed out that the membrane beneath the eggshell is an intrinsic biological component that plays a role in the normal physiological functions of the embryo [9, 43]. The soft PDMS interior cannot imitate the exact biological components present within the eggshell and, therefore, will only partially simulate its functions [9]. All in all, the benefits listed far outweigh the drawbacks, and as existing techniques evolve, the PDMS ex-ovo culture system will continue to remain relevant in current research practices and a go-to option for the choice of model.

Calcium Supplementation

Regarding shell-less ex-ovo cultures, it is imperative to replicate the functions of the eggshell through artificial means. These functions include acting as a physical barrier from the external environment, facilitating gaseous exchange, reducing water loss, and providing a vital calcium source for embryogenesis and developmental growth [114, 115]. Amongst the functions, calcium supplementation is one of the primary factors that significantly affect chicken embryo viability and hatchability [40]. The natural extraembryonic calcium sources can be derived from the yolk and the eggshell in domestic chicken, respectively [116]. According to several articles, the yolk contains about 20-25 mg of calcium, 3-4 mg in the albumen and the eggshell comprises roughly 2-3 g of calcium carbonate content [116-118]. Distinctively, during the early stages of embryonic development up to day 10 of incubation, the yolk remains the primary calcium source for the chicken embryo [40]. However, shortly after ten days of development, that role will be shifted to the eggshell via calcium absorption through the chorioallantoic membrane (CAM), whereby the translocation of eggshell calcium continues to contribute to the supply in the embryo and yolk [40, 43, 96]. Calcium transport

via the CAM is a highly regulated process, and the presence of the eggshell is an active component at both the biochemical and cellular levels [43].

Further analysis also revealed that calcium-binding protein (CaBP) plays a role in the transportation process as it is highly expressed in the inactive form within the CAM itself [41, 119, 120]. In addition, the studies have identified that calcium deficiencies and skeletal defects were common in long-term shell-less cultures [41, 119, 120]. Hence, these findings strongly suggest that direct calcium uptake and regulation from the eggshell are significant. One paper found that with the inclusion of calcium supplementation in a shell-less quail embryo culture, hatchability can be increased up to 40-50% [36]. Similarly, Kamihara et al. (1998) also reported an estimated 43% hatchability rate of quail embryos using calcium lactate as a viable supplement [121]. Other researchers have also described increasing survivability and hatchability rates for chicken embryos in ex-ovo culture systems with this addition [25, 26, 43].

Several different components can be considered for the usage of calcium supplementation. There are several factors to consider before deciding on the method of application. These include the type, quantity, purpose, and timing. The easiest method would be to utilize the eggshell itself by breaking it into smaller pieces or crushing it into a fine powder. This is common for commercial and industrial purposes, ranging from dietary supplement products to fertilizers and additives [122-124]. As the eggshell is primarily composed of calcium carbonate, it would serve as a decent source of calcium for embryonic growth [125]. However, there are multiple concerns with this method. First, calcium carbonate is inorganic, which is almost insoluble in water (solubility: 1.5 mg/100 mL-H2O at 25°C) [126]. This means that directly adding the powder or eggshell pieces to the albumen in the presence of distilled water might not be enough to provide sufficient absorption. Two other studies acknowledged this, which indicated that adding calcium carbonate to the albumen was not substantial enough to elicit a hatchability response [19, 36]. As a result, the timing of the supplementation is further

evaluated; it is known that the embryo derives its calcium from the eggshell through the CAM [43, 96]. Thus, it would validate the idea of adding calcium carbonate directly to the CAM for absorption. Tahara et al. (2020) experimented and found out that when 250 mg of calcium carbonate was added on days 5, 10, and 15, hatchability and survivability of the embryos were the highest for day 10, followed by day 15 [40]. This is likely attributed to the CAM being more fully developed on days 10 & 15 to absorb the calcium carbonate than on day 5 [40]. Other examples of additives that were investigated were calcium lactate and calcium chloride. Calcium chloride was not a viable option as it produced a fatal toxic effect observed in the embryos, usually after day 1 [36].

On the other hand, calcium lactate, which has low water solubility and relatively low toxicity, was considered a secondary alternative. When varying amounts of calcium lactate were added to the albumen, it was discovered that a high hatchability rate could be achieved using 25mg compared to the other quantities (0 mg, 10 mg, 20 mg, 30mg, 35mg, 45mg) [36]. Another paper reported that instead of adding calcium lactate to the albumen, it was added directly to the CAM. The result was that calcium lactate exhibited a toxic effect on the cells of the CAM, and all the chicken embryos did not survive the next day [40]. All the different methods discussed (fine eggshell powder, calcium carbonate powder, calcium lactate) can be utilized as an additive for calcium supplementation. However, the experimental conditions and varying control factors must be considered to maximize the success rate of chicken embryonic development.

Materials and Methods

Production of Bioengineered PDMS Eggshell

The transparent eggshell was manufactured using a heat-curable polydimethylsiloxane polymer (Sylgard-184, Dow Corning, USA). The PDMS kit comprises two components; the crosslinker, the curing agent, and the prepolymer, which acts as a base. In this study, the weight combination ratio utilized for the PDMS mixture follows the manufacture protocol recommendation of 10:1, with a total mixture volume of 18.1 ml. The mixture volume was taken from an existing paper bioengineering three-dimensional PDMS eggshells, where different mixing volumes and ratios were explored [27]. Ishak eventually discovered that the optimum range for the 10:1 ratio is between 15.4 to 18.7 ml of volume, where the PDMS produced had a uniform thickness and provided enough rigidity [27]. Once the crosslinker and the prepolymer were combined at the desired ratio, the mixture was stirred thoroughly to encourage homogeneity before being poured into an aluminum mold. It is then subjected to a vacuum chamber at 0.1 MPa for 1.5 hours to remove any unwanted air bubbles that may affect the integrity of the PDMS eggshell.

***Figure 6**: Procedure for curing and preparing the PDMS. A) Image depicting the type of prepolymer and curing agent used (Sylgard-184, Dow Corning, USA). B) Curing agent and prepolymer are mixed thoroughly according to manufacturer's ratio C) Mixture is placed within a vacuum chamber to remove unwanted air bubbles D) Image of polymer mixture before degassing E) Image of polymer mixture after degassing.*

The aluminum mold is a crucial component specifically designed for the fabrication of the PDMS eggshell to mirror the elliptical geometry of an egg. It comprises three attachable parts: the mold cover and two halves of the mold core. The mold is necessary for the fabrication process as it contributes to the uniformity of the eggshell via rotational movement throughout the curing process. Once the mold is fully assembled with the mixture inside it, it will be secured onto a loading platform on a multimodal biaxial bioreactor (TisXell Regeneration System) before being exposed to a parameter setting of 3:1 (major to minor axis speed) rotational speed ratio for 24 hours. Twenty-four hours is required for the mixture to be fully cured and uniformly distributed around the mold's inner surface. Once the curing process has been completed, the mold is disassembled, and the PDMS eggshell is removed with care.

Failure to adhere to the 24 hours requirement by removing either too early, too late, or utilizing different mixture ratios outside the recommended range may result in the PDMS eggshell tearing during the disassembly.

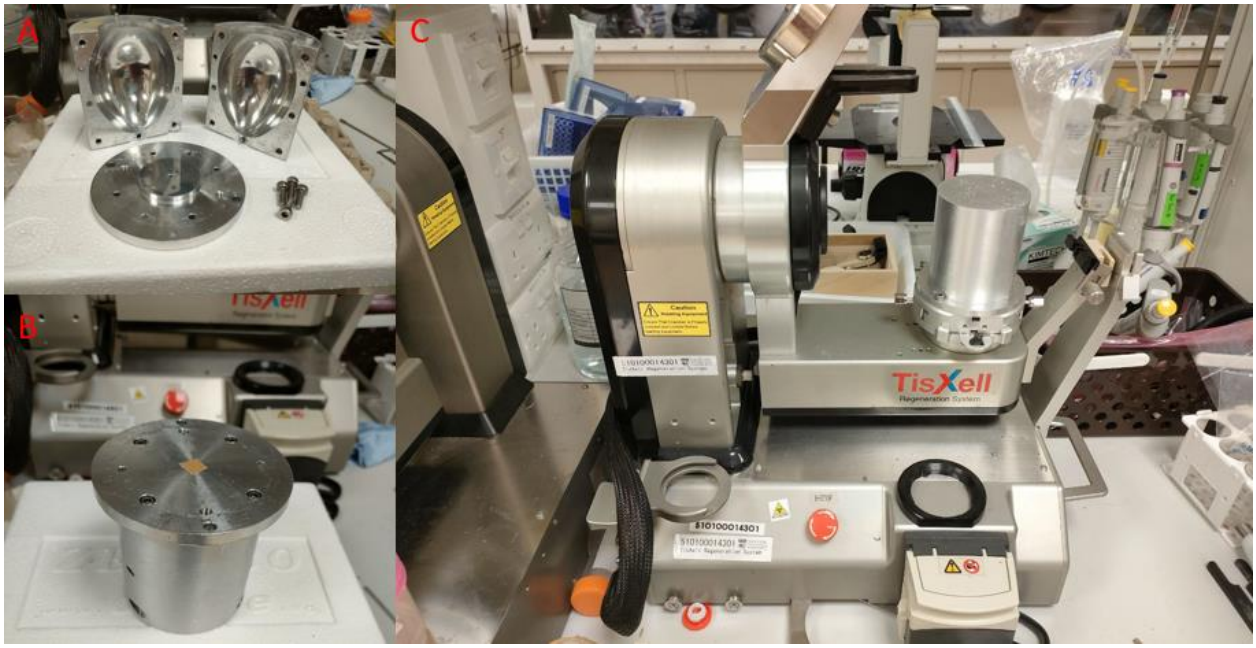


Figure 7*: Fabrication process of the ex-ovo PDMS model part one. A) Aluminum mold used to generate the PDMS shape and hold the mixture. B) Image of the assembled mold with PDMS solution in it. C) Complete setup of the biaxial bioreactor with the assembled mold.*

The realized product can be seen in Figure 6, where the mixture distribution and curing process fully sealed it. An opening is made at the top of the transparent eggshell using a minor surgical knife or scissor, which is essential for creating an entrance for the embryo transfer. In the existing literature review for PDMS models, a plastic film or sheet was used to enclose the opening to minimize external contamination. Ishak used a thin PDMS membrane of a 1 mm thickness to cover the gap before using additional PDMS to ensure complete adherence [27]. These applications are simple and easy to perform but fare poorer in other aspects such as sustainability. Hence, another alternative approach was adopted; using a reusable PDMS cover made from the bioengineered eggshell that can be removed easily. The entire fabrication

process of the cover is described below. Two raw PDMS eggshell models are required; one serves as the primary medium containing the egg's contents, and the other functions as a cover. The mixture ratio follows the eggshell mentioned above fabrication, with parameters of a 10:1 ratio at 18 ml. Although seemingly counterintuitive to forfeit one PDMS shell to create a cover, existing PDMS models that have become worn out can be recycled for this particular use case, making this approach more sustainable and efficient in terms of recyclability.

***Figure 8**: Fabrication process of the ex-ovo PDMS model part two. Requires two PDMS shells for this procedure. A) Raw product obtained after curing treatment and bioreactor process. B) The top of the first PDMS shell is cut off to facilitate an opening for the cover. C) A similar incision is made for the second shell, and it is placed in a petri dish with the same mixture formulation. D) Shell with the newly formed cover component is removed after curing. E) Top portion of the second shell is separated from the body to form a small, cap-like structure, which serves as a cover. F) Cover is placed on top of the first shell to conclude the assembled model.*

Cultivation of Chicken Embryo Culture and Setup

The fertile chicken eggs (Leghorn X Golden Comet) were obtained from an approved local poultry egg supplier in Singapore and subsequently placed in an incubator (Ova-Easy 380 Advance Series II Cabinet Incubator, Brinsea, USA) after a brief storage period for 56 hours at 37.5°C with 60% relative humidity before experiment commencement. The eggs were also conditioned to turn at a 45° angle clockwise and anti-clockwise with 30 minutes intervals throughout the entire process. Positioning the fertile eggs was small to end down to facilitate the air space at the top to remove the shell later on during embryonic transfer. Regarding ethical guidelines for using chicken embryo models in Singapore, Institutional Animal Care and Use Committee (IACUC) approval are unnecessary as this study's experimental aim, and objectives only require up to day 18 of embryonic development. Hence, IACUC approval is only mandatory if hatching is to be observed.

The entire experimental set is split into five sample groups: control and four test groups with varying calcium quantities. Each test group indicates supplementation at different junctures throughout the experiment. The calcium is introduced either on the day of the embryonic transfer (Day 3) or on (Day 10) for absorption. This setup is influenced by existing studies, whereby calcium supplementation was provided on Day 10, where the CAM is reported to be fully developed to extract calcium from the eggshell [40]. Sample harvesting, extraction, and imaging modalities were performed throughout the experimental period on specific time points (Days 3, 5, 8, 10, 12, 15, 18). Surviving embryos were terminated on Day 18 in alignment with IACUC compliance regarding non-hatching protocols. Figure 7 below describes the entire process and workflow of the program:

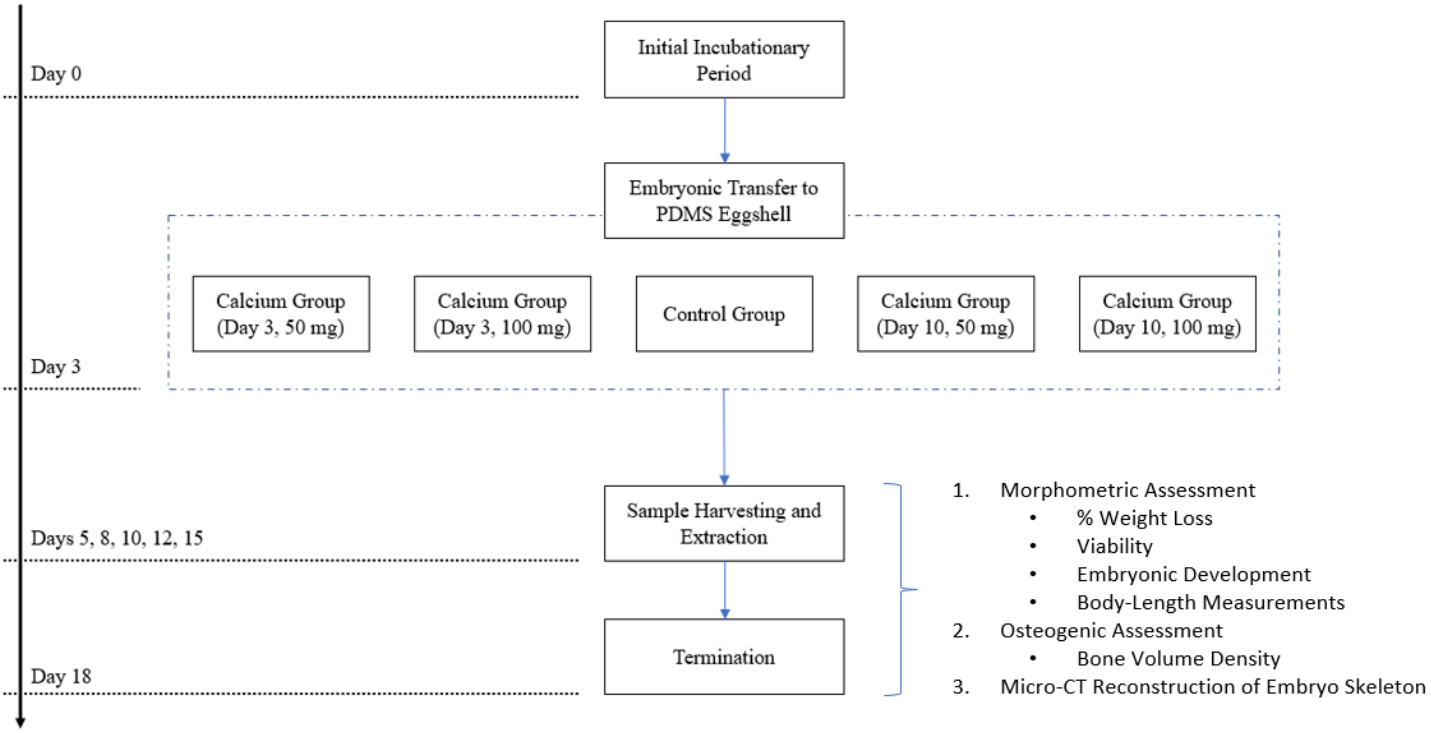


***Figure 9**: Flowchart describing the process throughout the experimental period starting from Day 0 to Day 18. Samples are split into five groups: (Control), (Day 3, 50mg), (Day 3, 100mg), (Day 10, 50mg) and (Day 10, 100mg). Experimental phases outlined are days 3, 5, 8, 10, 12, 15, 17, and 18. Corresponding analysis protocols were performed during these specific time points until termination.*

Transfer of the egg contents to the artificial eggshell is done under aseptic conditions to minimize contamination. Before the transfer day, PDMS eggshells and covers were sterilized in an autoclave machine (Autoclave Hirayama HV-85). The entire process is carried out in a biosafety cabinet (Gelman Singapore), where the eggs were also sprayed with 70% ethanol as a layer one disinfection to reduce surface contaminants. Layer two disinfection involved using povidone-iodine to wipe the exterior shell surface. Afterward, a small needle gauge (PrecisionGlide, 1.2mm x 38mm) was utilized to puncture a small hole in the lower half portion of the eggshell before attaching a syringe (Terumo, five cc/ml) to the needle and draining 5 ml worth of albumen out. This prevents the egg's contents from overflowing and spilling when the top shell is removed. Next, an eggshell opener made out of a dome, a metal pole, and a mobile rod is employed to carefully demarcate the upper shell portion and crack it into a perfect circle. The loose upper shell can then be gently removed using a pair of forceps or scissors to detach the piece entirely, revealing the egg's contents alongside the embryo. The embryo constituents

are then poured into the PDMS eggshell before adding the 5 ml of albumen and closing the gap opening with the cover. It is then placed back into the incubator for cultivation.

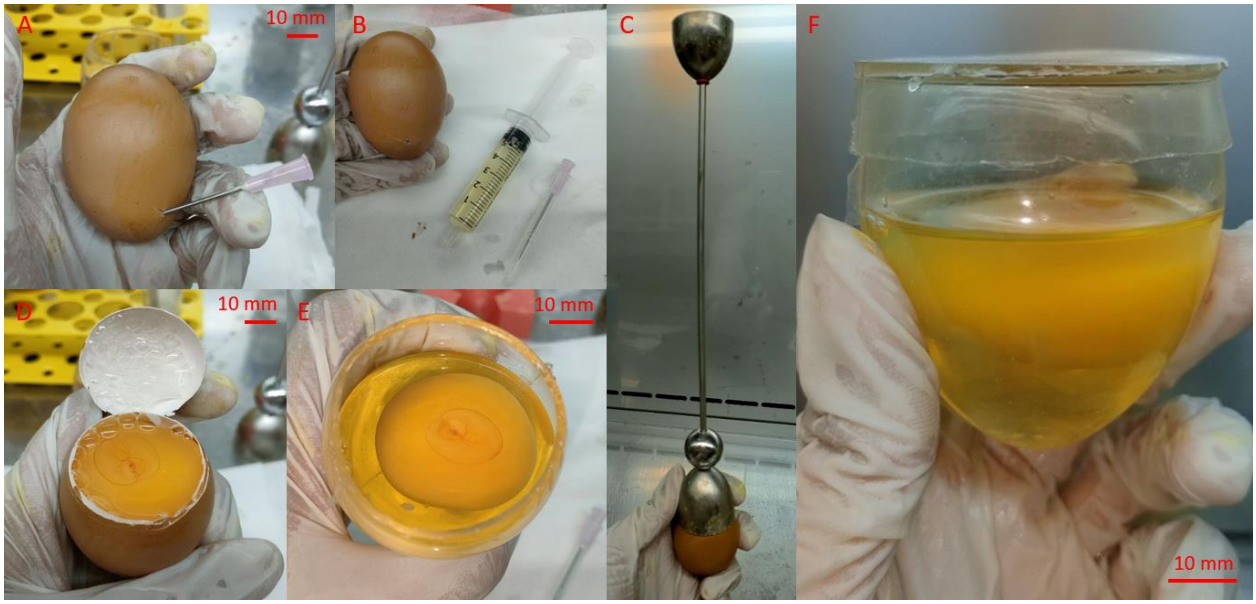


***Figure 10**: Image describing the embryonic transfer process. A) After disinfecting the egg with ethanol and iodine, a small perforation is made at the bottom to facilitate albumen extraction. B) 5ml of albumen is extracted to reduce chances of overflow after cracking. C) Access to the embryo is carried out using an eggshell opener. D) The cracked upper shell is removed to expose the embryo and the egg constituents. E) Contents of the egg are carefully transferred to the PDMS eggshell before adding the 5ml of albumen extracted. F) The opening of the PDMS shell is sealed with the cover and marks the final step of the embryonic transfer.*

Calcium Supplementation and Procedure

Chicken eggshells were used in this study as the primary means of calcium supplementation for the embryo. Several factors supported the decision to utilize eggshell powder for this particular experiment. Industrial usage of combining feed with crushed eggshells for supplementing chicken dietary needs has long been implemented to boost overall egg quality and improve bone mineralization [124, 127]. In addition, other essential trace minerals found in the eggshell, such as magnesium, phosphorus, copper, zinc, manganese, and iron, are also essential to the embryo's development [128, 129]. Parallel studies also indicate that chicken eggshells are a viable calcium supplement for human consumption [122, 123].

Empty eggshells obtained post-embryonic transfer were cleaned thoroughly before crushing using a pestle and mortar. Next, the eggshells were ground to a fine powder and stored in a glassware container sent for sterilization via autoclaving to reduce any remaining pathogens. The added calcium powder is dependent on the experimental group, either on the day of the transfer (Day 3) or on Day 10. For Day 3 calcium supplementation, the powder was deposited onto the bottom of the PDMS eggshell before transferring the albumen and the rest of the egg contents. On Day 10, the calcium addition is carried out by gentling and depositing the powder onto the edges of the chorioallantoic membrane in a crescent-like manner. Supplementation is done in a sterile environment (biosafety cabinet) to reduce the chances of contact contamination. This process takes about 20 seconds, and the embryo is immediately returned to the incubator within a one-minute timeframe to minimize any homeostatic disruption such as prolonged humidity or temperature changes.

It is essential to differentiate the two supplementation methods pertaining to this experiment. Adding calcium on Day 10 stems from the knowledge that from Day 1 to 9, the chicken embryo mainly sources its calcium needs from the yolk and albumen [40, 41, 43]. During this period, the CAM begins to develop and proliferate, where Day 10 of incubation

commonly characterizes the complete maturation of the CAM, which the embryo will then begin to source its extraembryonic needs from the eggshell [41]. Thus, administering calcium from this point onwards will ensure that the embryo can continue fulfilling its mineral requirements. Conversely, augmenting the embryo with calcium on Day 3 has also been a viable alternative among researchers. Several forms of calcium additives, such as calcium lactate, calcium carbonate, eggshell powder, and calcium salts, have been experimented with by depositing them into the culture vessel before embryonic transfer [36, 40].

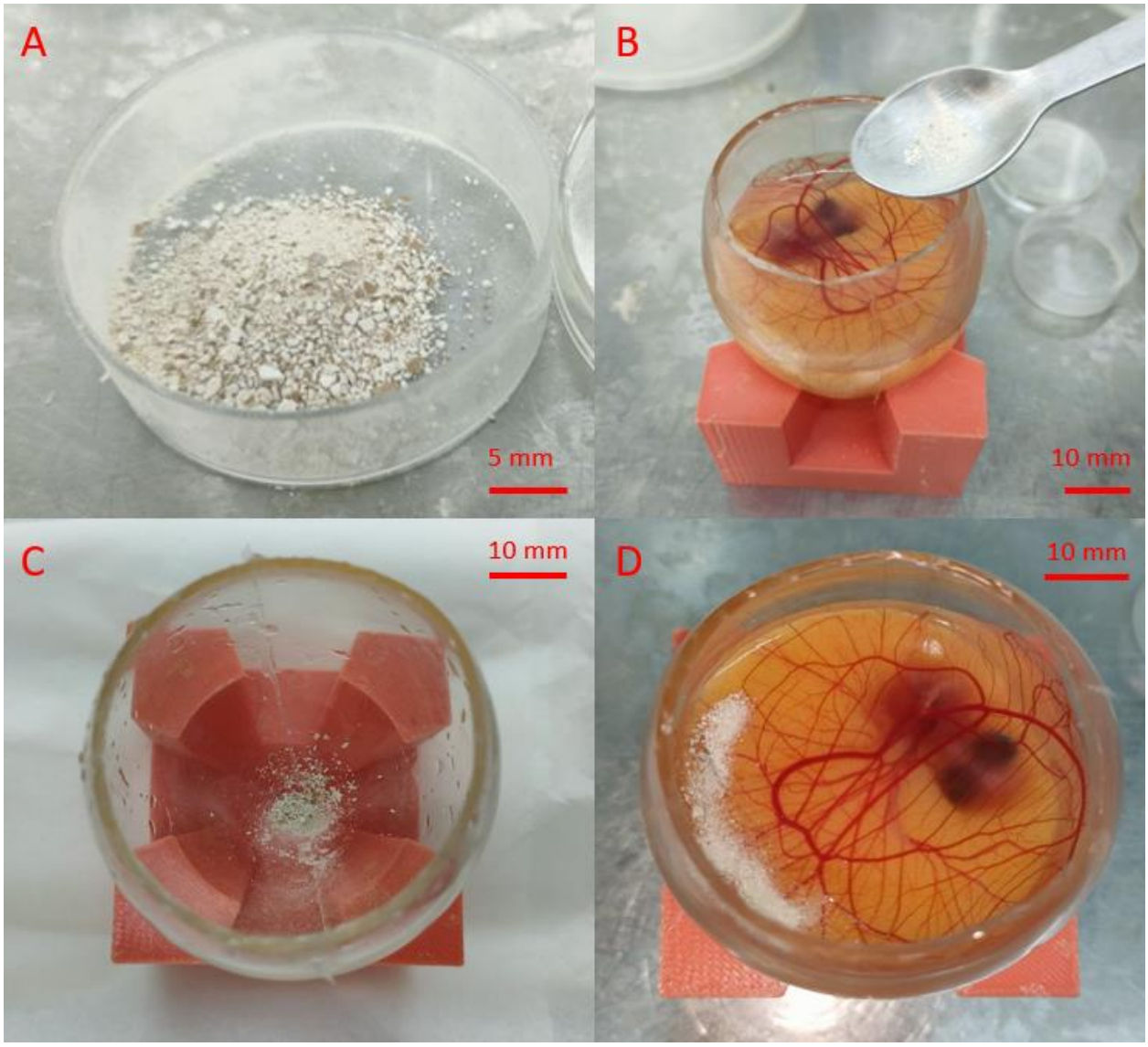


***Figure 11**: Description of the calcium supplementation procedure for two main types of test groups; Day 3 and Day 10. A) Primary form of calcium powder supplementation is obtained from crushed eggshells. B) Calcium powder is added onto the CAM for Day 10 groups. C) Calcium powder is added onto the bottom of the PDMS eggshell for Day 3 groups. D) For Day 10 groups, the powder is deposited towards the edge of the CAM, close to the PDMS eggshell.*

Sample Harvesting and Measurement

The termination period for the chicken embryo is on Day 18, whereby the chicks were euthanized by placing them in a -20°C fridge for two to three hours. After which, the frozen chick is allowed to thaw before being extracted from the PDMS eggshell using forceps and scissors. Morphological measurements were taken before the chick was washed using distilled water, cleaned, and then placed in formalin solution (Sigma Aldrich) for 24 hours or more in preparation for a micro-CT scan for bone density evaluation. Samples attained per group for each specific timepoint were ensured to meet the minimum statistical requirement (n = 3) for evaluation.

Embryonic Development

For gross observation of chicken embryonic development, ex-ovo imaging was performed using a handheld smartphone (Oppo Reno 2) that captured the biological developments at various developmental stages over the entire experimental period.

Weight Loss Assessment

To determine the overall aspect of healthy growth and development, the weights of the chicken embryos were constantly monitored. Weight loss progression of the embryos was measured as a percentage of the initial starting weights after embryonic transfer from egg to PDMS eggshell. The weight of the embryos was recorded on the experimental timepoint periods (days 3, 5, 8, 10, 12, 15, 18) using a digital weighing machine. Box plots were used to compare the effect of calcium supplementation on % weight loss at day 18 in embryonic development. One-way ANOVA was also performed to evaluate statistical significance amongst the treatment groups.

Viability Assessment

The viability of the chicken embryos was evaluated to understand the influence of calcium supplementation in ex-ovo PDMS eggshells. It was evaluated based on the length of survival for 18 days. Viability timepoints were identical to the experimental time points of days 3, 5, 8, 10, 12, 15, and 18. The number of chicken embryos that survived up to different time points and the corresponding survivability rate were calculated based on the total sample size deployed for every particular experiment group.

Morphometric Assessment

Morphological analysis was carried out on Day 18 after the chicken embryo had been euthanized. Once the embryo was isolated from the yolk sac, chorioallantoic membrane, and the amniotic fluid, respective measurements of the embryo's primary characteristics (beak, body, toe) were taken using threads. This form of analysis can be taken from Hamilton et al., who recommended using this set of characteristics as deterministic factors for overall development observation [130]. First, as a general guideline, the beak length was calculated from the tip to the base of the eye socket, and the toe length was determined using the third toe from phalanx one to four. Next, the whole-body length was evaluated from the tip of the beak around the body circumference to the third toe. Once that is completed, the threads are aligned with a ruler to ascertain the lengths in centimeters. Finally, statistical evaluation was performed using Welch’s t-test to determine any substantial relevance between the test groups.

Osteogenic Assessment

Qualitative and quantitative bone examination is crucial in determining the influence of calcium supplementation in ex-ovo models. Therefore, osteogenic assessment in bone volume density analysis and bone reconstruction of the chicken embryo was introduced via micro-computed tomography (CT) scan.

Micro-CT Reconstruction of Embryo Skeleton

The chicken embryos were removed from the formalin solution, dried, and wrapped in parafilm before interacting with the micro-CT scanning equipment (InspeXio SMX-90CT). Before each scanning sequence, the embryo is positioned on a platform with radiopaque clay in a fetal arrangement, featuring the natural embryonic position in an egg. The equipment's software is then used to adjust the platform's scanning parameters to optimal before commencement. Images captured were then transferred to a separate computer loaded with analysis software (VG Studio) for further analysis. A deterministic surface setting (background, material, ISO value) was applied to differentiate bone material characteristics from biological tissue. The specifications are as follow: background: 35626; material: 65927; ISO value: 50537.

Bone Volume Density Analysis

Each sample's morphometric properties were calculated according to the above specifications, based on the overall bone density volume within a specified region of interest. Next, the computed results were averaged and compiled into a table respectively. Finally, additional statistical analysis was performed using Welch’s t-test to determine any observable significance.

Results

Summary Table of Sample Sizes in Experiments

Table 3: List of sample sizes utilized for each experiment

Type of Analysis	Number of Samples Utilized (n =)
Embryonic Development	**20** for each group
Weight Loss Percentage	**3-4** for each group
Viability Percentage	Control: **64** D3, 50mg: **20** D3, 100mg: **28** D10, 50mg: **34** D10, 100mg: **43**
Morphometric Indicator	**4-5** for each group
3D Reconstruction of Embryo Skeleton	**3** for each group
Bone Density Volume	**3** for each group

The sample sizes obtained for each analysis can be observed in the summary (Table 3) above. There is a large difference of sample sizes obtained especially for the viability percentage analysis. To clarify, the reason why there is a large range of sample sizes for each group is because of a statistical need to obtain a minimal significant value (n = 3 to 5), especially for the Control group. Considering the fact that the viability percentage for the Control group on Day 18 is the lowest, it was difficult to obtain at least (n = 3) on the last timepoint when there is a high mortality rate for Control embryos between Day 12 onwards. This is also true for other sample groups such as (D10, 50mg) and (D10, 100mg), which required more samples in order to obtain a statistically meaningful value for later timepoints such as Day 15 and Day 18.

Sample Analysis of Embryonic Growth and Development

Embryonic Development

Snapshots for each group were taken at specific time points (Day 3, 5, 8, 10, 12, 15, 18) to record the embryonic progress from experiment initiation to termination. Images were taken for the Day 10 (50mg, 100mg) calcium groups comprise only the second week (Day 10, 12, 15, 18) as calcium supplementation was only introduced on embryonic day ten onwards. In summary, most test groups presented natural growth patterns characteristic of a healthy chicken embryo, except for the Control and Calcium (Day 10, 100mg) groups on Day 18. Both groups cited the existence of a white substance floating in the allantois beneath the CAM, as shown in the images (Figure 10, 14). This is in stark contrast to the other attained samples, where this particular component was absent in the Day 18 images (Figures 11, 12, 13). Postmortem diagnosis and cross-examination confirmed that this white substance is associated with feather expression and said feathers on the embryos. As observed in Figures 10 and 14, the related embryos presented naked bodies with little to no indication of feather growth or attachment. In terms of visible morphological landmarks, when comparing across all test groups, little to no significant variation was detected, as the developmental phases reflect the stages referenced in the HH model and are generally in sync.

Control Group

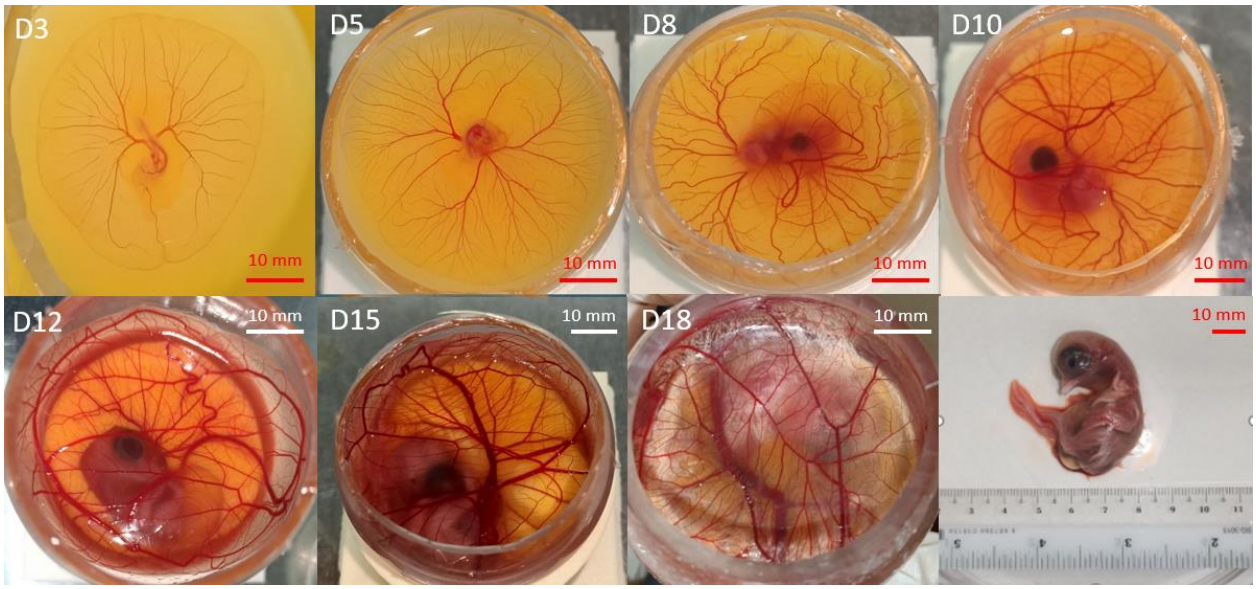


Figure 12*: Series of chick embryonic development for Control Group (0 mg). Specimens in this group (n = 4) exhibited average growth with major developmental milestones achieved (proper cranial development, limb formation, presence of beak tooth, and feather germination). However, Day 18 indicated signs of feather anomaly with the presence of a white substance in allantois. Post-mortem analysis revealed that the substance was likely to be feather constituents.*

The image above (Figure 10) represents an example of the evolutionary process of the chicken embryos acting as the control and not supplemented with any form of calcium. The sample demonstrated typical growth developmental patterns (limbs, eye socket, beak) in accordance with the HH model from the start of the experiment to the termination. However, a post-mortem examination revealed that the embryo is ill-developed, evidenced by the lack of feather germination and production in several body areas. A closer look on Day 18 also unveiled numerous white, string-like substances floating in the allantois beneath the CAM. Further examination revealed that this unknown matter was likely to be feather constituents, as some of this substance can be observed coming off from the embryo itself.

Calcium Group (Day 3, 50 mg)

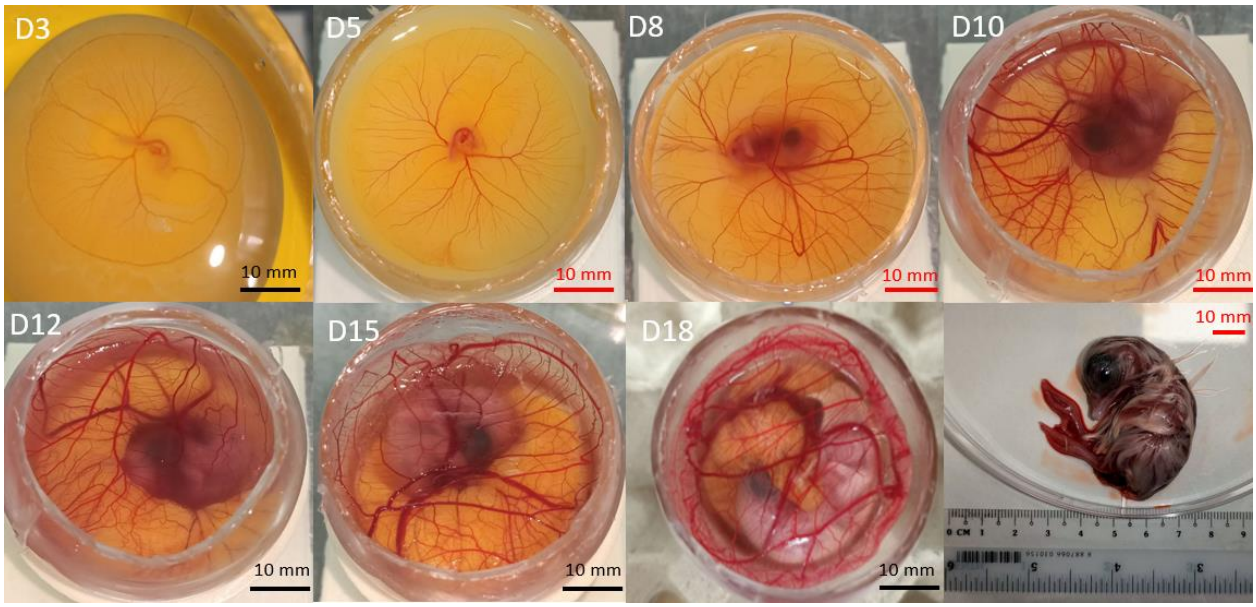


***Figure 13**: Series of chick embryonic development for Calcium Group (Day 3, 50 mg). Specimens in this group (n = 4) exhibited average growth with major developmental milestones achieved (proper cranial development, limb formation, presence of beak tooth, and feather germination).*

The illustration above (Figure 11) depicts an example of the different staging processes for chicken embryos supplemented with 50mg of calcium on Day 3. The sample displayed normal progression in its development, evident by the presence of specific morphological landmarks (limbs, eye sockets, feathers, beak). In addition, the different stages detailed in accordance with the HH model can be observed throughout the specific time points. No variation in proper growth structure or abnormalities were noted.

Calcium Group (Day 10, 50 mg)

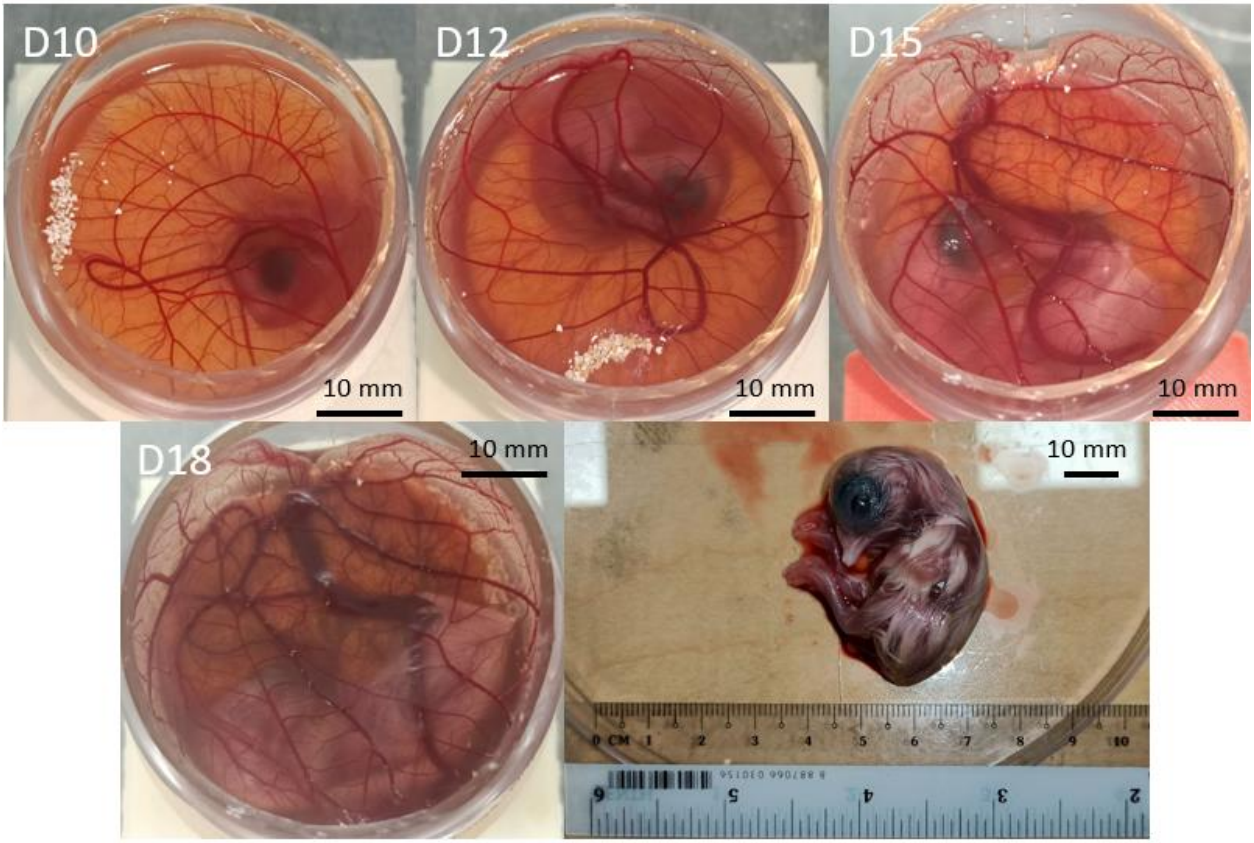


Figure 14*: Series of chicken embryonic development for Calcium Group (Day 10, 50 mg). Specimens in this group (n = 5) exhibited average growth with major developmental milestones achieved (proper cranial development, limb formation, presence of beak tooth, and feather germination).*

Similarly, with the previous Calcium groups aforementioned above, it can be observed in Figure 12 that the chicken embryo supplemented with 50mg of calcium on Day 10 presented signs of natural growth, with minimal indication of any abnormalities. These signs include morphological landmarks such as proper limb formation, beak pipping, feather growth, and eye pigmentation. Regarding the interaction between the calcium powder and CAM, no adverse reaction was noted in terms of toxicity or antiangiogenic effects. In addition, the calcium powder has also seemingly assimilated with the CAM after a few days since its inception.

Calcium Group (Day 3, 100 mg)

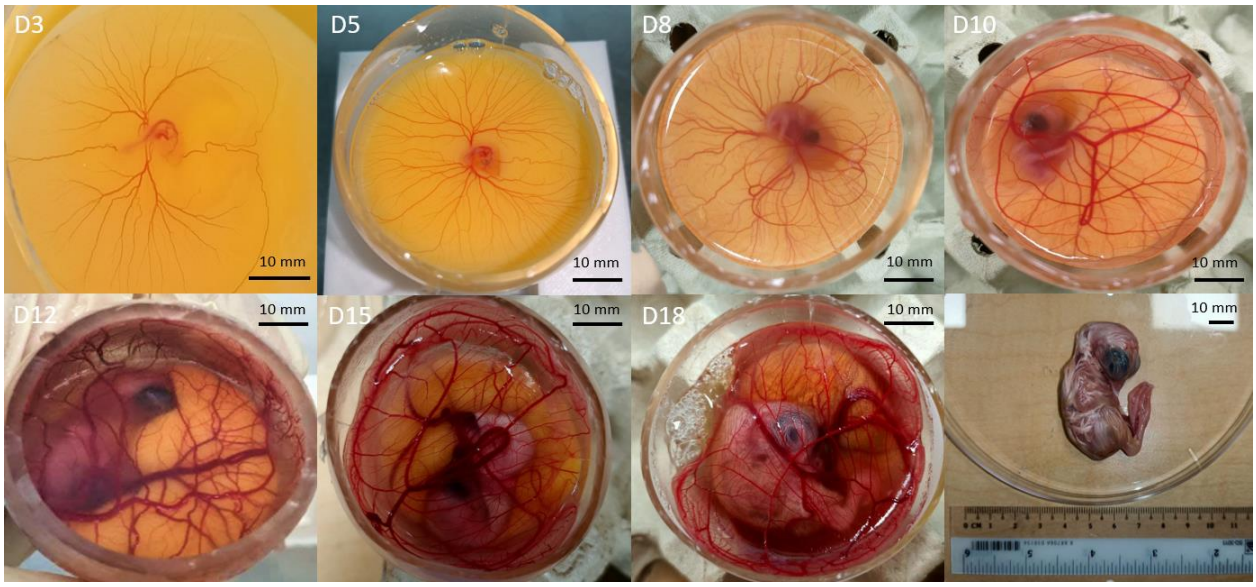


Figure 15*: Series of chicken embryonic development for Calcium Group (Day 3, 100 mg). Specimens in this group (n = 5) exhibited average growth with major developmental milestones achieved (proper cranial development, limb formation, presence of beak tooth, and feather germination).*

For the chicken embryos supplemented with 100mg of calcium on Day 3, the samples exhibited growth and development characteristics of a normal embryo. The embryonic phases captured during each specific timepoint were also compared with the referenced HH stages. Referring to the sample illustrated in the figure above (Figure 13), no significant abnormalities were present, and significant morphological landmarks (limbs, feathers, beak, eye formation) can be detected during post-mortem examination.

Calcium Group (Day 10, 100 mg)

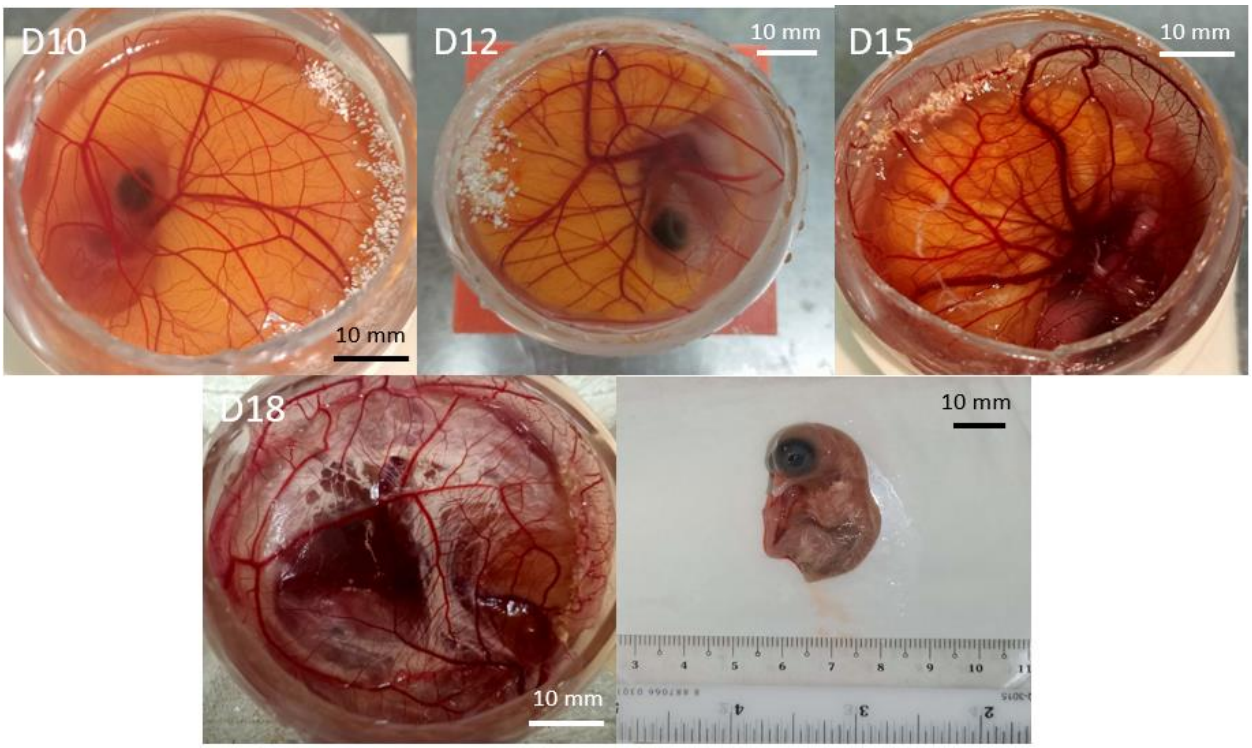


Figure 16*: Series of chicken embryonic development for Calcium Group (Day 10, 100 mg). Specimens in this group (n = 4) exhibited average growth with most of the major developmental milestones achieved (proper cranial development, limb formation, presence of beak tooth, and feather germination). However, Day 18 indicated signs of feather anomaly with a white substance in allantois. Post-mortem analysis revealed that the substance was likely feather constituents.*

Figure 14 encompasses an example of the developmental process from Day 10 to Day 18 for chicken embryos supplemented with 100mg of calcium on Day 10. A first glance, the embryo demonstrated expected growth with minimal variation from Day 10 to 15, and the calcium powder produced no visible adverse reaction. However, Day 18 also reported a drastic deviation from healthy maturation regarding an unknown white matter present in the allantois beneath the CAM. Upon closer inspection, it was revealed that the white substance might be feather constituents, which were coming off the embryo body. Post-mortem analysis confirmed the deduction as the embryo depicted above showed no indication of feather growth or any signs of feather attachment on the body.

Weight Loss Percentage

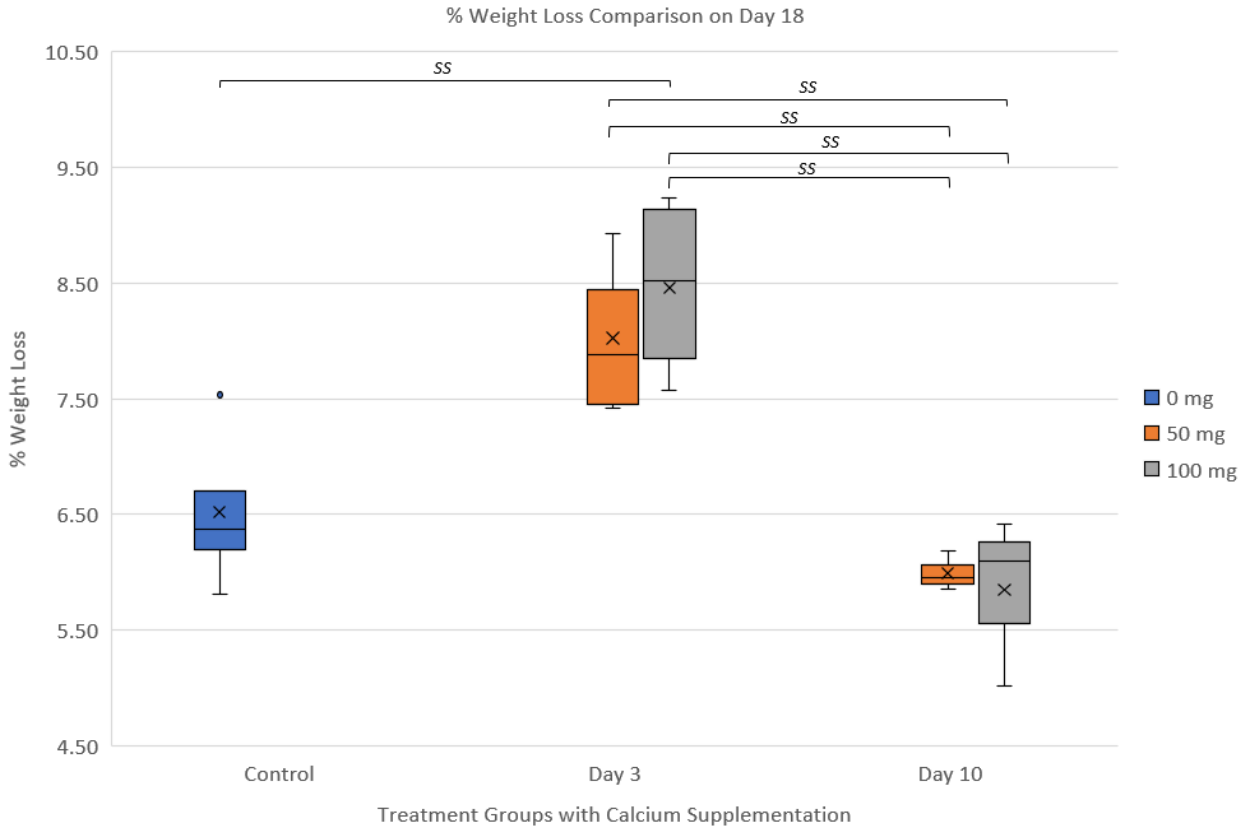


***Figure 17**: Box plot comparison analysis for weight loss percentages was obtained for all test groups based on Day 18 results. Test groups supplemented with calcium (50mg, 100mg) on Day 3 exhibited the highest weight loss percentages, whereas test groups supplemented with calcium (50mg, 100mg) on Day 10 displayed the lowest values. A one-way between subjects ANOVA was conducted to compare the treatment groups based on percentage weight loss amongst the treatment groups, with a significant effect at $p < 0.0005$ level. Post-hoc Tukey HSD test was also carried out to determine statistical significance between treatment groups.*

Figure 17 shows the box plots for each treatment groups (Control, Day 3, Day 10) on Day 18 against percentage weight loss as a function of the calcium quantity (0mg, 50mg, 100mg) supplemented. Based on the figure, the median percentage weight loss for the Control was 6.32%, whereas the median percentage weight loss for the 50mg and 100mg calcium groups ranged from 6.10% to 7.93%. The Control group also presented a single outlier in the data. It was also observed that the Day 3 calcium groups exhibited higher percentage weight loss than both the Control and Day 10 groups. Due to multiple comparisons between two or more test groups, one-way ANOVA was conducted to compare the effect of calcium on percentage

weight loss in chicken embryos for the selected treatment groups. There was a significant effect of the treatment groups on percentage weight loss remembered at the $p < 0.05$ level for the following conditions: [$F(4, 13) = 10.54$, $p = 0.000496$]. As a statistically significant result was present in the findings, Tukey post-hoc test was conducted to determine the relationship between treatment groups. Post-hoc comparisons revealed that the (Day 3, 50mg) was statistically different than both Day 10 groups, and (Day 3, 100mg) was statistically different than the Day 10 and Control groups. Taken together, the results suggest that significantly higher percentage weight loss may be observed for Day 3 calcium groups as compared to Day 10 calcium groups.

Viability Percentage

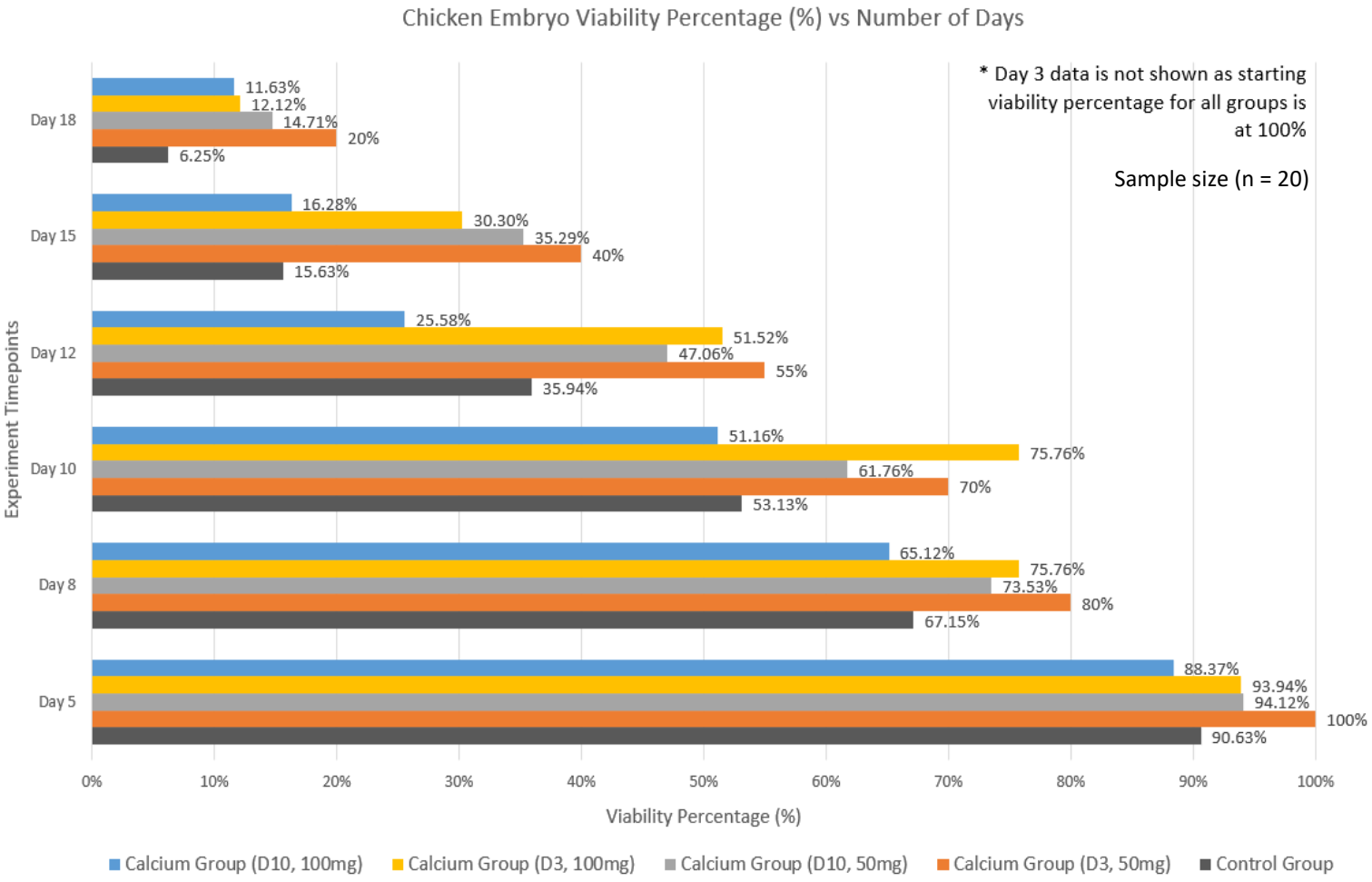


***Figure 18**: Catergorical comparison analysis for viability percentages obtained across all test groups. Data was acquired over a period of 18 days on specific time points (days 3, 5, 8, 10, 12, 15, 18) and compiled as a derivative of the total number of chicken embryos utilized for each group. The raw information obtained was classified as binary values (dead/alive), according to whether the embryo has survived up till specific timepoints. All test groups supplemented with calcium exhibited higher viability percentages than the control group (6.25%), with (Day 3, 50mg) Calcium group attaining the highest value at 20%. In addition, test groups supplemented with 50mg of calcium performed better than test groups supplemented with 100mg of calcium, at 20%, 14.71% and 12.12%, 11.63% respectively.*

Figure 18 depicts the embryonic viability study conducted for the experimental groups over the entire duration. This section analyzed basic information acquired as binary data (dead or alive). Survival rates were calculated as the number of remaining chicken embryos surviving up to specific timepoints over the total sample size available per group. Values tabulated and shown in the figure are percentages across the timepoints denoted in varying colors. As

perceived from the results above, the Control group yielded the lowest viability at the end of the experimental period on Day 18, with a total percentage rate of 6.25%. Compared to the calcium groups, the resultant viability was almost half that of the 100mg Calcium groups (D3, D10) at 12.12% and 11.63%, respectively, indicating that groups supplemented with calcium had higher efficacy.

On the other hand, (D3, 50mg) Calcium group exhibited the highest viability yield at 20% on Day 18, making it the best performing category amongst all the test groups. Comparing the 50mg Calcium groups (D3, D10) and 100mg Calcium groups (D3, D10), it can be noted that the viability percentages for embryos supplemented with 50mg of calcium (20%, 14.71%) fared better than those added with 100mg of calcium (12.12%, 11.63%). In addition, it is evident that for 50mg calcium supplementation, the Day 3 group (20%) has a greater viability rate than the Day 10 group (14.71%). Between the two other 100mg Calcium groups (Day 3, Day 10), minimal variation in percentage rates can be observed on Day 18.

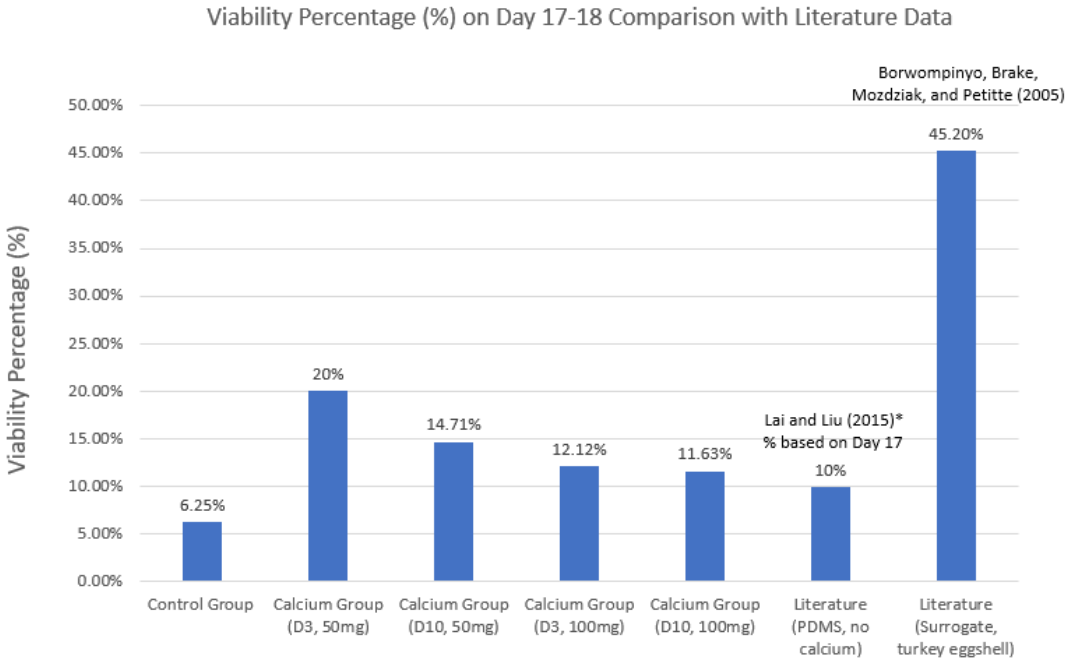
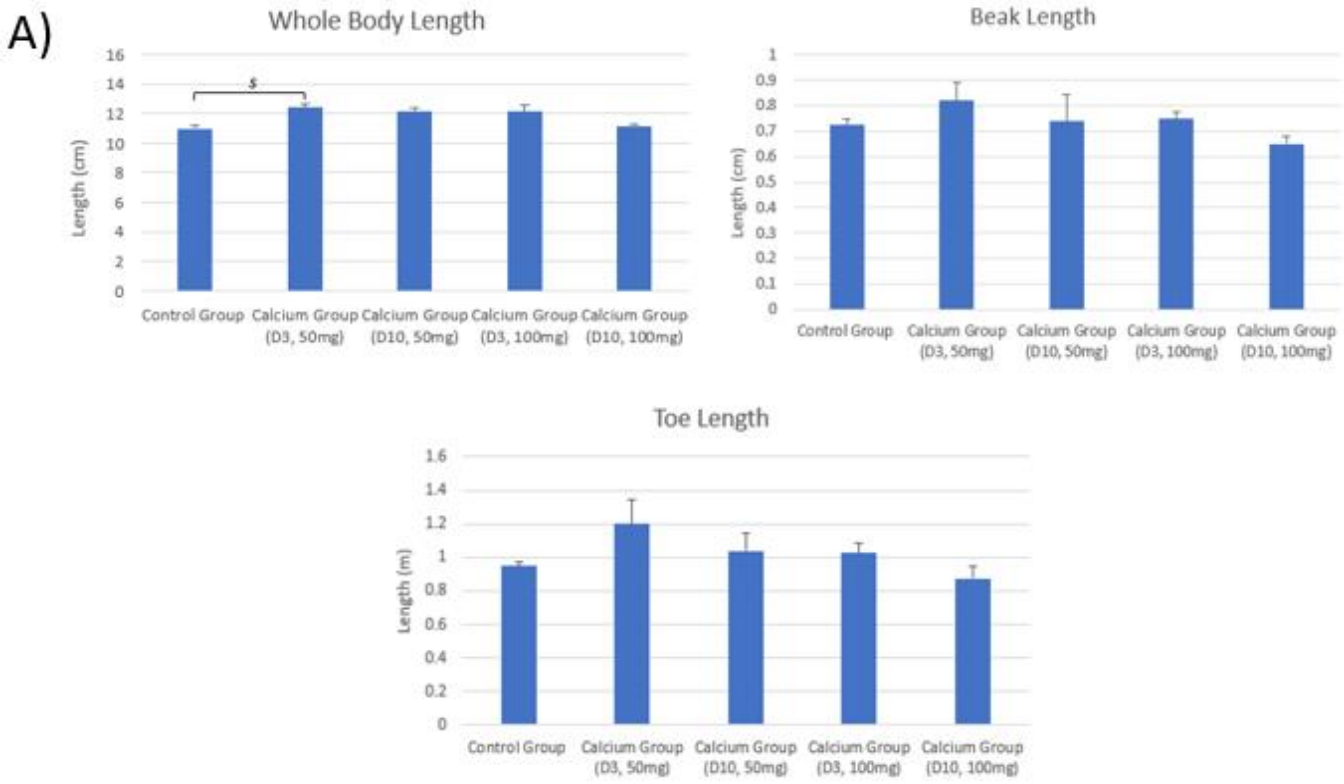


***Figure 19**: Comparison of Day 17-18 viability percentages with existing ex-ovo models. Literature data was extracted from two separate studies involving the utilization of a cuboid PDMS model and a surrogate turkey eggshell model.*

A direct comparison with reference to existing ex-ovo and in-ovo models as shown in the figure above. In order to provide a fairer approximate analysis without including other variables such as oxygen aeration or calcium type for example, literature data has been extracted from two separate studies; one involving the usage of a cuboid PDMS ex-ovo model without calcium supplementation to cultivate chicken embryos [9], and another utilizing surrogate turkey eggshells to host the embryos [131]. It can then be observed that for the literature in-ovo surrogate model, it produced a 45.2% survivability rate on Day 18 as compared to the literature ex-ovo PDMS cuboid model, which produced a 10% on Day 17 instead. There was no Day 18 data available for the PDMS cuboid model.

Morphometric Indicator



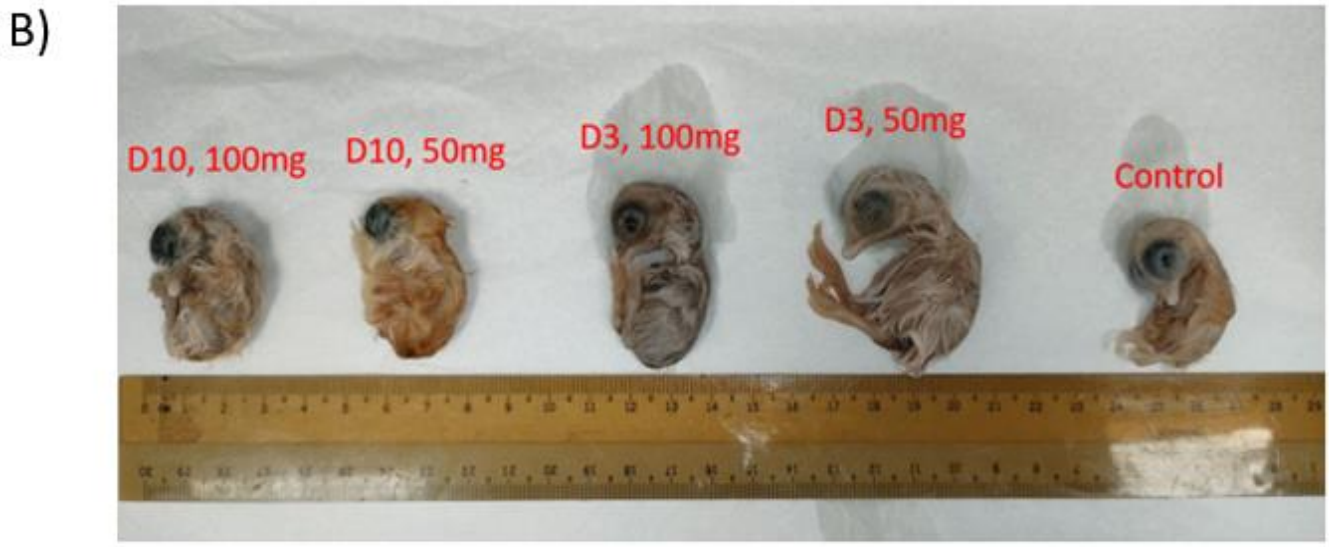


***Figure 20**: Comparison analysis for morphological measurements obtained across all test groups. A) Data was acquired at point of termination on Day 18 and assessed on a basic of three metrics: whole body length, beak length, and toe length. A one-way between subjects ANOVA was conducted to compare the treatment groups based on respective morphometrics, with a significant effect at $p < 0.05$ level for whole-body length measurements. Error bars are displayed accordingly (standard error). Post-hoc Tukey HSD test was also carried out to determine statistical significance between treatment groups. B) Representative image of embryos obtained across all test groups. Based on gross observation, the Control embryo presented the smallest in overall body size, whereas the (Day 3, 50mg)*

embryo possessed the largest body size as compared to the others. No observable difference can be made for the other test groups.

Morphological measurements (beak, toe, whole-body) taken from the chicken embryo on Day 18 were analyzed and compiled into Figure 20. The corresponding length (cm) of the anatomical parts was plotted against the experiment groups, and any statistical relevance calculated is shown above. Due to multiple comparisons between two or more test groups, one-way ANOVA was conducted to compare the respective morphometric measurements in chicken embryos for the selected treatment groups. For toe length measurements, there was not a significant effect of the treatment groups at the $p < 0.05$ level for the following conditions: [$F(4, 17) = 1.29$, $p = 0.312$]. For beak length meaasurements, there was also not a significant effect of the treatment groups at the $p < 0.05$ level for the following conditions: [$F(4, 17) = 0.76$, $p = 0.565$]. As for whole-body length measurements, there was a significant effect of the treatment groups at the $p < 0.05$ level for the following conditions: [$F(4, 17) = 4.3$, $p = 0.013$]. As a statistically significant result was present in the findings, Tukey post-hoc test was conducted to determine the relationship between treatment groups for whole-body length results. Post-hoc comparisons revealed that the (Day 3, 50mg) was statistically different than the Control group. This is indicative of the representative image shown in B), which depicts a larger embryo from the (D3, 50mg) group and a much smaller embryo from the Control group. It is also worth noting that the (D3, 50mg) embryo is much larger than the other embryos from the remaining calcium groups. No other observable differences can be acquired from the other samples in this category.

Sample Analysis of Embryonic Bone Development

3D Reconstruction of Chicken Embryo Skeleton

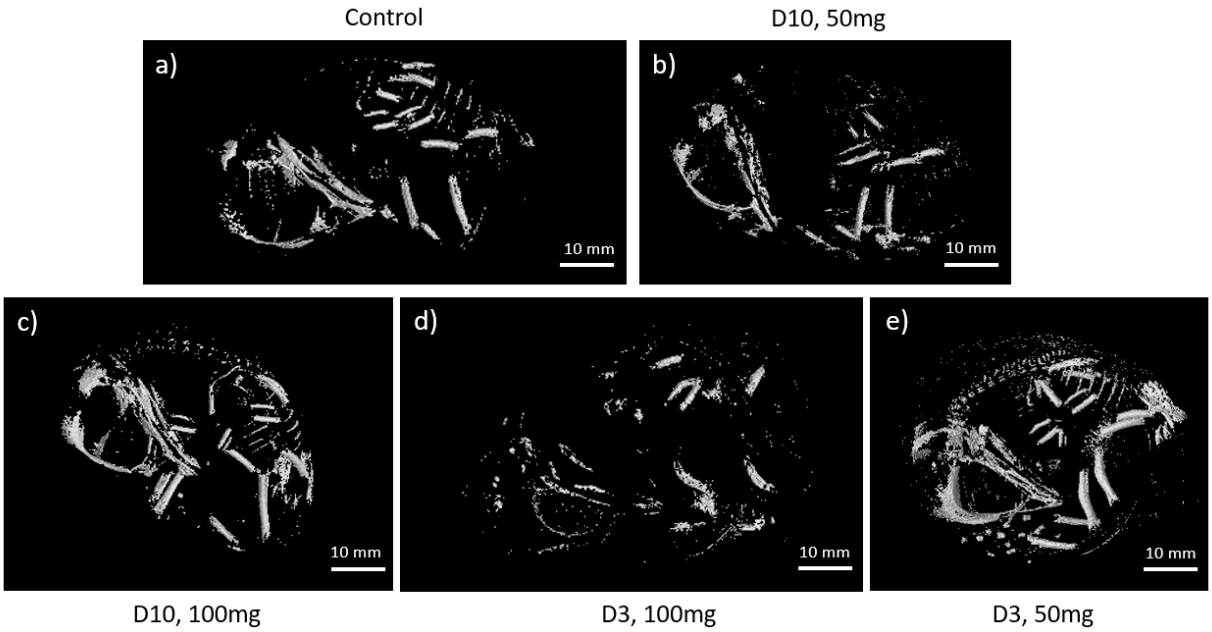


***Figure 21**: Micro-CT reconstruction of the chicken embryo skeleton on Day 18 for (a) Control group, (b) (Day 10, 50mg) Calcium group, (c) (Day 10, 100mg) Calcium group, (d) (Day 3, 100mg) Calcium group, (e) (Day 3, 50mg) Calcium group. Visible cranial and vertebral structures can be observed in images (c) and (e), whereas sparse spatial bone islands can be noted for images (a), (b), and (d).*

Figure 21 depicts the chicken embryo skeleton and bone formation visualization for samples across all test groups via micro-CT imaging and three-dimensional reconstructions. As observed, defined cranial, vertebral, coastal, and limb structures can be identified for embryonic images c) and e), and a) to a certain extent. On the other hand, sparse spatial bone islands were noted in the positions of the skull, vertebrae, and limbs for embryonic images b) and d).

Bone Density Volume

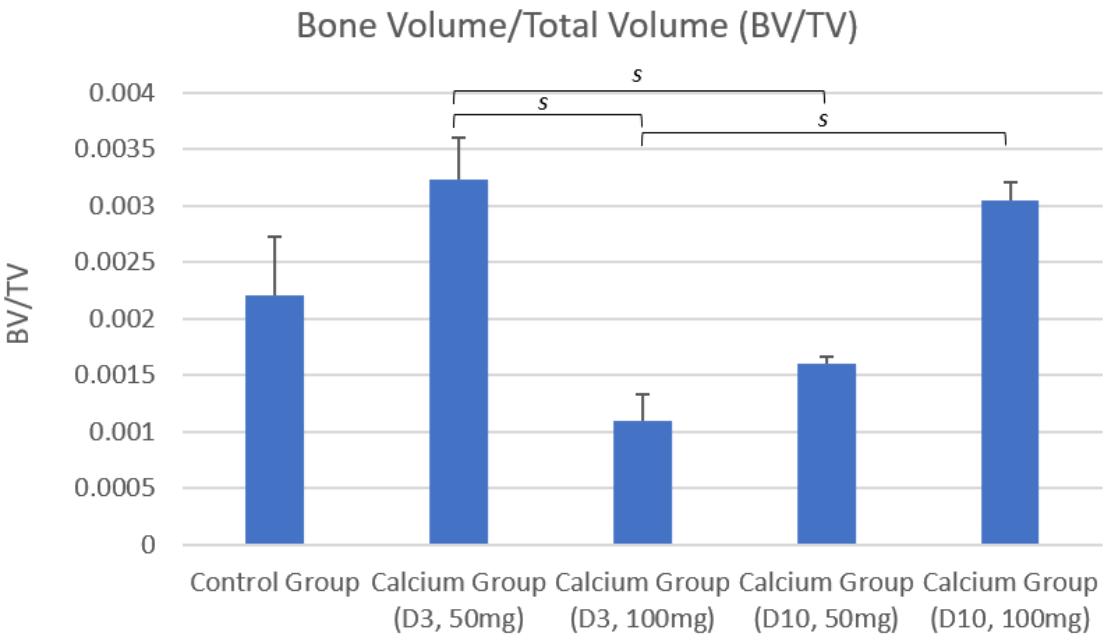


***Figure 22**: Comparison analysis for bone volume versus total volume of chicken embryo samples across all test groups with error bars displayed (standard error). Data was acquired on Day 18 post-embryonic termination after micro-CT analysis. A one-way between subjects ANOVA was conducted to compare the treatment groups based on bone volume to total volume ratio, with a significant effect at $p < 0.005$ level. Post-hoc Tukey HSD test was also carried out to determine statistical significance between treatment groups.*

In Figure 22, data was acquired by specifying an area of interest with pre-determined specifications to evaluate each test group's bone density morphometric parameters for embryonic samples. Due to multiple comparisons between two or more test groups, one-way ANOVA was conducted to compare the effect of calcium on bone volume to total volume ratio in chicken embryos for the selected treatment groups. There was a significant effect of the treatment groups on bone volume to total volume ratio remembered at the $p < 0.05$ level for the following conditions: [$F(4, 10) = 8.5$, $p = 0.002$]. As a statistically significant result was present in the findings, Tukey post-hoc test was conducted to determine the relationship between treatment groups. Post-hoc comparisons revealed that the (Day 3, 50mg) was statistically different than both (Day 3, 100mg) and (Day 10, 50mg) groups, and (Day 3,

100mg) was statistically different against (Day 10, 100mg) group. Taken together, the results suggest that significantly higher bone volume to total volume ratio may be observed for (Day 3, 50mg) and (Day 10, 100mg) calcium groups as compared to the remaining calcium supplemented groups.

Discussion

The primary purpose of this study was to investigate the effects of external calcium supply on the biomimetic PDMS eggshell model as an ex-ovo chicken embryo model. Specifically, the effects of calcium supplementation on embryonic growth and development and overall bone development were examined. These goals were achieved via differing the quantity of calcium supplied (0mg vs. 50mg vs. 100mg) and its application on different embryonic time points (Day 3 vs. Day 10). The qualitative and quantitative results suggested that external calcium influence encompassed both beneficial and detrimental components, and the significance of each aspect is interdependent.

Adverse Effects on the Absence of External Calcium Supply

It is essential to assess if calcium is a necessary component for the healthy growth and development of chicken embryos and the roles that it might play in ex-ovo culture systems. Specifically, in chicken embryos, the importance of calcium has long been touted as a significant contributor to proper bone development, regulating extraembryonic functions, and supporting overall embryonic growth [37, 40, 41, 132]. Furthermore, calcium deficiency in ex-ovo systems has been widely studied to understand its impact on metabolic pathways and transport mechanisms [41, 43]. Several papers have elaborated this further in the past, including the manifestation of calcium-deficient induced retarded growth and embryogenesis of embryos in shell-less cultures [39, 42]. Hence, upon evaluating the embryonic developmental results attained, it can be ascertained that the absence of supplemented calcium does elicit a negative effect on specific characteristic patterns of the embryo, particularly concerning its viability rate and feather growth.

The indication of feather malformities on embryos is rather conspicuous when pertaining to the Control group in Figure 12. Numerous factors revolve around feather growth

and loss, including hormonal balance, genetics, infectious agents, environment, and nutritional diet [133]. Since the samples acquired were of the same species and culture methods were largely standardized, it is unlikely that anything other than the addition of calcium would affect the results obtained in the experiment. Therefore, a possible explanation is that an imbalance in trace mineral composition in egg constituents might have affected calcium homeostasis and perturbed other regulatory mechanisms responsible for the growth and germination of the embryo feathers. A paper written by Leeson and Walsh supports this notion, stating that inadequate levels of trace minerals present in embryonic contents may disrupt feather development [133]. Another plausible reasoning can also be attributed to the role of calcium in tissue morphogenesis and early chicken skin development. Li et al. discovered that voltage-gated calcium channels and ions are primarily responsible for coordinating feather mesenchymal movements and promoting feather bud elongation [134]. This is supplemented by another study involving regional specificity skin research, mentioning that voltage-gated calcium signaling may play a part in feather skin development [135]. Therefore, an absence of said external calcium supply may contribute to the defective elements of feather growth and germination.

The results speak for themselves regarding the viability rate, with Figure 18 exhibiting only a 6.25% survivability rate on Day 18 for chicken embryos in the Control group. Calcium is a necessary component for healthy embryonic growth [37, 128], and the deprivation of this mineral source will have several implications, including overall poor growth and calcium-deficient-related abnormalities. One study also reported an increase in mortality rates for calcium deficient chicken embryos [39]. Coupled with the fact that this systemic absence also likely contributes to defective feather growth [133], this further supports the rationale that the lack of a calcium supply negatively impacts the chicken embryo.

Beneficial Influence of External Calcium Supply

Pertaining to the influence of calcium in embryonic development, it is without question that calcium as a metabolic component is integral and necessary for avian embryos to function correctly before and after hatching [36, 43]. Cultured chicken embryos growing in a shell-less, calcium-deficient environment often experience developmental abnormalities and increased mortality rates [40]. Scientists apply this knowledge as a form of evaluation in ex-ovo models when isolating calcium-related studies such as transport mechanisms [41], bone development [42], and calcium-specific expressions [134]. In other cases, researchers also regard this as a means to improve and optimize ex-ovo research; the quality of embryonic growth remains relevant when planning for long-term goals regarding ex-ovo studies. Thus, understanding why and how external calcium affects embryonic growth and development is incredibly beneficial and would aid in prolonging the efficiency of ex-ovo avian models. With that being said, investigations revealed that calcium supplementation did have an overall beneficial effect on the growth and bone development of the chicken embryos. This was particularly evident in several convoluting aspects:

1. Significantly enhanced percentage weight loss progression was observed for some Calcium groups (D3, 50mg & D3, 100mg) in Figure 17. In order to understand the importance of using weight loss percentages as a metric, its involvement with healthy embryonic development must be elucidated. A healthy, typical chick hatched after 21 days typically generates an average weight loss of about 11 to 12% of its initial value [136]. This gradual change throughout its incubation can be attributed primarily to the metabolic activity throughout the embryonic developmental phase. During its growth stage, for every gram of fat utilized for metabolic processes, an equivalent amount of water is also generated [137]. The water product is then steadily removed over time via diffusion through the eggshell to the external environment [137]. Hence, there is a correlation between weight loss and embryonic

growth regarding healthy chick development. Given that the Day 3 Calcium groups (50mg, 100mg) attained weight loss values of 8.02% and 8.46%, respectively, on Day 18, this trend is on par with that of a normal chicken embryo development, indicating a healthier growth when compared to the Control and other Calcium groups. Indeed, the interrelationship between calcium, embryonic metabolism, and weight loss may be established simply because elemental calcium contributes significantly to the health and development of the chicken embryo [41, 43, 116]; it is only logical to consider that subtracting the availability of calcium would derive a negative response in terms of impaired development and hence, reducing metabolic capabilities.

2. Increased viability percentages were observed for all Calcium groups compared to the Control on Day 18 in Figure 18 It is no surprise considering the substantiality that calcium is a vital component of healthy growth and impacts the viability of these chicken embryos [132, 138]. Tahara et al. performed a similar experiment whereby several test groups were subjected to different parameters of calcium supplementation. It was found that no hatching from the control group was observed, whereas embryos in the other test groups supplied with calcium experienced hatching and increased survivability [40]. In addition, other parallel studies also reported an increase in overall viability for avian embryos when calcium supplementation was introduced [25, 36]. These were coherent with the results obtained in this experiment, observing a 2 to 3 times increase in viability rates as compared to the Control, supporting the notion that external calcium supply positively affects the embryo's ability to survive in ex-ovo culture.

3. Significantly heightened bone density was reported for some Calcium groups (D3, 50mg & D10, 100mg) in Figure 21. Since calcium is necessary for bone mineralization and growth, hypocalcemia, bone stunting, and poor ossification are often reported in embryos without calcium supplementation [18, 39, 41]. Therefore, it is noticeable that some test groups supplemented with calcium produced favorable bone density growth compared to embryos that did not receive similar treatment. Furthermore, according to one study, primary bone

development and calcification are mainly reliant on calcium mobilization from the eggshell; the absence of this or any form of external calcium introduction will affect the embryo's growth characteristics [38]. In light of this, these factors may very well align with this study's findings.

4. Significant difference in whole-body length for (D3, 50mg) Calcium group compared to the Control in Figure 20. Regarding the larger whole-body size, this outcome is pretty within expectations, considering the nutritional importance of calcium. Furthermore, when the embryos are raised in a calcium-deficient environment, existing literature papers commonly observed periods of smaller dry mass and retarded growth [138, 139]. Therefore, it is also rational to correlate available embryonic size with overall skeletal formation. Furthermore, since calcium directly involves skeletal mineralization, facilitated growth with calcium availability would likely impact the length and size of the embryo's skeletal features, affecting the entire body length [43]. This means that a lack of major calcium sources would impede proper bone formation and probably result in an overall decrease in body size due to poorer skeletal structure [27].

Quantity and Time-Dependence of Calcium Supplementation

Apart from considering the other two developmental insights aforementioned above, discerning how supplementing calcium at different timepoints and varying its quantity will affect the growth and performance of the embryos is also of utmost importance. The distinction between the Calcium test groups in terms of expected results for development, percentage weight loss, viability, and bone density volume proves that this subject cannot be discounted. The primary takeaway can then be derived based on the previous statement; the effects of external calcium supply on the chicken embryos are dose and time-dependent. This is perceptible in the following pointers:

1. Not only did the embryos from the Control group present feather abnormalities on Day 18, but embryos from the (D10, 100mg) Calcium group also produced similar results in Figures 12 and 16. This is crucial, considering that only these two groups displayed such a phenomenon; the other test groups were regular. This then bodes the question as to why having some or none of the calcium supplementations still evokes the same detrimental response. It has already been established that without calcium, the risk of abnormal development is high, noticeable by the results gathered from the Control group. Consequently, when taking a comparative look at the Day 10 Calcium groups (50mg, 100mg) in Figures 14 and 16, the former embryo (50mg) obtained did not exhibit the same symptoms as that of the latter (100mg). An insight could then be drawn from this piece of information; seemingly contradictory, higher quantities of calcium than lower quantities may be more substantial in producing adverse effects inflicted upon the embryo. Referencing a parallel study conducted in 1959, scientists determined that high calcium levels used in the experimental dietary program for young pheasants resulted in overall poor growth, whereas this effect was reversed when less calcium was implemented [140]. Furthermore, another paper remarked that excess calcium in avian birds leads to elemental zinc imbalance, essential for preventing parakeratosis [141].

Parakeratosis is a skin condition that induces hyperkeratinization of epithelial cells, which may be further exacerbated with levels of surplus calcium [141, 142]. As such, there exists a possibility that utilizing more significant calcium quantities such as 100mg as compared to 50mg shown in the experiment may lead to the outcome observed through its influence.

2. Embryos with 50mg calcium addition on Day 3 and 10 developed lower mortality rates as a function of higher viability at the end of Day 18 compared to embryos supplemented with 100mg on both days in Figure 18. Likewise, with the above observations, utilizing a higher quantity of calcium may not equate to a more significant beneficial effect on the chicken embryos. In one paper, researchers discovered that excess calcium absorption via a larger dosage might lead to undesirable effects in avian embryos, such as corresponding increased mortality [140]. Consequently, this was relatively consistent with another research that adopted calcium carbonate (CaCO3) as a primary form of supplementation. Tahara et al. verified this theory by demonstrating that when comparing 250mg versus 350mg of CaCO3 supplementation, embryonic hatchability was reduced for the latter [40]. However, it must not be forgotten that although the findings statement suggests a lower quantity might be more efficient when compared to each other, the (D3, 50mg) Calcium group had higher viability percentages (20%) than the (D10, 50mg) Calcium group (14.71%). This also implies that supplementing calcium earlier on embryonic Day 3 versus Day 10 may be a better alternative. A study revealed that chicken embryos yielded an overall higher viability rate when an identical amount of CaCO3 was added on Day 3 instead of Day 10 [40]. This discovery corresponds well to the above, meaning that apart from considering that supplying calcium on Day 3 may be a better alternative than Day 10, the quantity is also a factorial role in defining the overall survivability yield.

3. Supplementing calcium (50mg, 100mg) on embryonic Day 3 yielded significantly higher weight loss percentages than those supplemented on Day 10 in Figure 17. That being

said, it raises another potential question; why were Day 10 test groups the worst-performing category in terms of lowest mean percentage weight loss and even lower than that of the Control group? Assuming the above holds and knowing that calcium is highly involved in general metabolic processes [143], one possible implication is that the calcium absorption via Day 10 was not well facilitated enough to foster metabolic needs. A conceivable reason could be the bioavailability of calcium presented to the embryo on both days of supplementation. For embryos supplemented with calcium on Day 10, Tuan previously reported that shell-less embryos experienced significantly lower calcium-binding protein (CaBP) and transport activities in the CAM than normal embryos [41]. CaBP is a high molecular weight protein that is primarily described to be implicated in numerous calcium-mediated processes such as facilitated transport, mobilization, and intracellular communication [119]. Tuan discovered that although the developmental expression of CaBP is present in shell-less cultures, levels of calcium-binding activity were stagnant because the CaBPs were not bioactive [41]. Another follow-up study revealed that this inactive form of CaBP could be rendered bioactive when the CAM is in direct contact with an eggshell piece [43]. Interestingly enough, the region of the CAM in contact with the eggshell expressed functional CaBP, whereas other regions that were not in contact denoted inactive CaBP expression [43]. Given how the supplemented method was performed (Figure 9), it is very likely that bioactive CaBP expression was diminished due to the limited surface area covered by the eggshell powder added on Day 10 to the CAM. This would probably affect the process of calcium uptake and in turn, reduce extraembryonic calcium supply for fetal development. Conversely, it could also be hypothesized that bioactive CaBP expression could be enhanced if a larger surface area of the CAM was covered by the eggshell powder, leading to increased calcium uptake. This yet remains to be seen.

As for the embryos with added calcium on Day 3, adding the eggshell powder to the albumen increased metabolic activity, and weight loss is still not well characterized. Most of

the existing research is dedicated to introducing calcium to the CAM, the primary facility for translocation and uptake between the embryo and the eggshell, not the albumen [41, 43, 119]. In one instance, Tahara et al. formerly demonstrated that adding calcium lactate on shell-less embryonic day three culminated in higher hatchability than those not supplemented with any calcium but did not delve into its process [40]. As such, there exist a few possibilities in which this notion might be feasible. A recent paper in 2020 by Arena et al. evaluating the peptidomics analysis of hen egg-white also revealed the existence of novel putative bioactive molecules, several of which were calcium binding [144]. This creates a remote possibility that these molecules might have interacted with the eggshell powder and mobilized calcium ions for embryonic usage. Another likelihood might be attributed to the constituents of the albumen itself. Albumen from the typical domestic fowl contains about 88.5% water and 10.5% proteins, which these water sources and proteins are vital to the embryo's survival [145]. One of these predominant proteins is the ovalbumin, which functional properties are thought to be biological, such as anti-microbial and immune-modulating activities, although the exact biological function is unknown [146]. It has been indicated that ovalbumin can bind to a considerable amount of calcium ions through non-specific binding (high-capacity, low affinity) [147]. Coupled with the knowledge that albumen proteins can flow into the yolk sac, amniotic fluid, and the digestive tract of the embryo [145, 148], this signifies the prospect of a possible mechanistic uptake of calcium ions from the deposited eggshell powder to the embryo for consumption. Nonetheless, these rationales are not substantial enough, and further research is required to identify potential calcium-related mechanisms.

4. Significant distinction in bone volume density between (D3, 50mg) and (D10, 100mg), versus (D3, 100mg) and (D10, 50mg) Calcium groups in Figure 21. Interestingly, the (D3, 100mg) and (D10, 50mg) fared poorer than the Control group, despite a lack of statistical significance. This is also evident in Figure 20, whereby representative images from (D3,

100mg) and (D10, 50mg) displayed sparse bone islands and incomplete bone formation in several key areas such as the cranial, vertebral, and limb structures as compared to the Control. This is unlike the (D3, 50mg) and (D10, 100mg) Calcium groups, which presented visible and defined key skeletal structures in the positions of these key areas of interest. Based on these observations, it can then be deduced that the relationship between the dosage and time-dependence of calcium supplementation is intertwined. If adding 100mg of calcium on Day 3 was less beneficial than adding 50mg, why was this not appear in the Day 10 groups, where the 100mg outperformed the 50mg in bone density? As discussed previously, one possibility suggested for the disparity between the Day 10 groups is the surface area absorption of the CAM and bioavailability of the calcium ions [43, 119]. Since calcium is a direct component of bone mineralization and formation [38], there is a direct correlation. As for the Day 3 groups, it has already been established that excess calcium in avian embryos is detrimental to the overall growth and well-being [96, 140]; therefore, there is a real probability of attributing the empirical findings to this explanation. Moreover, as the CAM is primarily responsible for regulating calcium mobilization and uptake [96], one consideration may be that facilitation of calcium uptake via the bottom of the PDMS eggshell is unregulated due to the CAM's absence. Hence, it may explain the perceived response from the (D3, 100mg) group accordingly. Once again, other than the proposed justifications mentioned above, the real underlying reason for calcium action via albumen deposition remains to be seen. Further investigation is required in future experiments for a more unambiguous indication to account for viable options.

Calcium Bioavailability and Distribution

In this study, the bioavailability and distribution of calcium repletion through the eggshell powder was not investigated. Hence, in order to provide a rough estimate of the amount of calcium available via external supplementation, an existing study that analyzed elemental composition changes of chicken eggshell during its incubation period may be utilized as reference [149]. It was discovered in the paper that on the first day of incubation, the calcium content was roughly about 390mg/g [149]. This finding was also consistent with literature data with results of 386-415mg/g [149, 150]. Although the breed of the chicken does play a role in varying the calcium content of the eggshell, mineral composition analysis may also be carried out in future works in order to establish a baseline for the amount of external calcium supplied to the PDMS ex-ovo model. Based on this thesis methodologies and literature data, the supplemented initial calcium content can be estimated as follows: 19.5mg for 50mg deposition, and 39mg for 100mg deposition. It was also highlighted by Szeleszczuk et al. that on incubation day 21 for normal chicken embryos, the concentration of calcium in the eggshell fell to around 332mg/g, roughly about 85% of the original amount [149]. From this, we may approximate that the calcium mobilization quantity to be around 58mg/g, and since the supplementation quantity used in this experiment is lower than that (19.5mg, 39mg), it may be reasonable to assume that majority of the external calcium supplied will be mobilized. Naturally, this would require a more in-depth investigation to validate the approximation of calcium bioavailability and distribution within this model.

Challenges and Limitations

Despite the results and insights attained, it is also essential to acknowledge any presence of limitations revolving around this study and understand the implications that follow. The first limitation is identifying feather abnormalities in the form of a white substance observed in the Control and (D10, 100mg) groups, which is based on gross observation of the phenomenon. Therefore, it is subjected to biases and confounding that may influence the development of vital insights. This is because no specific characteristic analysis was conducted to ascertain the actuality of the phenomenon, except for the fact that the white substance can be observed coming off from the embryo's body in post-mortem analysis. Still, other potential factors cannot be discounted, such as the prevalence of bacterial contamination. However, this phenomenon was recurring for multiple samples in the groups above, making the possibility of this phenomenon happening by chance unlikely. There are also existing studies suggesting why this incident may occur [133, 134, 140]. No similar incidents were reported in previous studies regarding calcium supplementation for ex-ovo models, making it challenging to obtain literature-based evidence. In addition, the focus of the majority of the studies was not to investigate solely on the comparative effects of calcium supplements between different embryonic time points but mainly on the calcium action with the CAM [40, 43, 96], having additional and contrasting parameters [25, 36, 40], or applying external calcium because existing literature review suggested it to be beneficial otherwise [26, 33].

The second limitation pertaining to the study is not accounting for the contribution of other trace minerals in the eggshell and shell membrane when evaluating the aspects of the results attained. According to the literature review, studies often attribute the eggshell to its primary component, a significant calcium source [9, 38]. As such, different constituents of calcium (eggshell powder, calcium carbonate powder, calcium lactate, and calcium carbonate nanoparticles) have been introduced to replace the function of the eggshell [36, 40, 43, 44].

However, considerations were not considered for potential factors, such as the presence of other trace minerals [128] or the functionality of the shell membrane [115]. Besides calcium, the eggshell and shell membrane is also known to contain other essential trace minerals such as copper, strontium, and boron, which play a vital role in supporting the embryo's healthy development [128, 151]. As such, even though it cannot be denied that the action of calcium in this context is the major contributor to the attained results, it is also inappropriate to entirely disregard any potential influence from the components above.

On the grounds of consideration, it must also be acknowledged that although external calcium supplementation was successful in enhancing several experimental aspects of this ex-ovo model, one must pay particular attention to the viability of the embryos, which undoubtedly is the most influential factor. This is because it is key to determining if the model system is suitable for short or longer-term studies, typically involving the embryos surviving for up to two weeks or more until hatching. In this study, notwithstanding the increased viability for all Calcium groups with the highest percentage obtained at 20% (D3, 50mg) on Day 18, the overall value is still relatively low to recognize this model as a suitable alternative for longer-term studies (> 14 days). This issue is not uncommon, as another study also reported similar observations with a 10-15% viability rate on Day 21 for embryos supplemented with calcium [40]. Using Figure 19 as reference, comparisons were also made with reference to in-ovo and ex-ovo literature data. From there, it can be noted that while the literature PDMS cuboid model exhibited a low viability percentage (10%) based on its Day 17 data, the surrogate model as a general representative of the in-ovo system boasted a very high viability rate (45.2%) as compared to the literature and ex-ovo groups in this experiment. This is because with the inclusion of a calcaemic shell, this would greatly raise the survival chances of the avian embryo for long-term development till hatching. This is readily apparent in several literature studies cultivating avian embryos in-ovo as well [23, 131, 152]. On the other hand, it can also be noted

that while the literature PDMS cuboid model technically has higher end-stage viability percentages than the PDMS Control in this experiment, it would be noteworthy to know that the 12-day survival rate (not shown) for the literature PDMS model is lower than that of the Control (27% vs 35.94%) [9]. This may also suggest that in terms of overall sustainability without considering calcium supplementation, this PDMS model may be more suitable in supporting avian embryonic life over a wider range of incubation development, but not till hatching. Generally that is not much of a concern, as most ex-ovo models are exploited typically for developmental studies and CAM-manipulation related research [26, 105, 153] before hatching, whereas in-ovo models may be the ones utilized for various hatching purposes [36, 154].

Supplementing onto the point above, it is also essential to acknowledge the importance of an extra-embryonic enrivonment, and that an absence in this enrivonment may results in adverse changes within the avian embryo physiological system, possibly altering its development and growth in ways that may be unknown. It is also no surprise that for the large majority of living beings, calcium regulation is key to numerous physiological functions and cell signalling [155]. Case in point, one example was reported by Miyahara et al. that cardiovascular anomalies related to systemic calcium deficiency were observed in shell-less chicken embryos and may be reduced by the addition of external calcium [156]. Another study also investigated the case of retarded embryos cultivated in ex-ovo cultures exhibiting lesser serum levels of insulin-like growth factor-I as compared to in-ovo embryos [157]. IGF-I has been suggested to be involved in regulation metabolism, growth, differentiation, and developmental events [158]. As such, while the action of biological signalling pathways specifically in PDMS ex-ovo systems remain to be investigated, these potential alterations exclusive to ex-ovo models cannot be discounted. Having said that, one may then begin to question the relevance of PDMS ex-ovo models as compared to conventional in-ovo such as

the windowed or surrogate models. Although in-ovo typically benefits more from greater survival rates and therefore, the possibility of extended cultivation periods, they are not the most conducive for time-lapse imaging studies, especially for later-stages of development [159]. This is because late-stage in-ovo studies are often impeded by the window and presence of developed extracellular membranes, making it difficult for embryonic manipulation and visibility [152]. In addition, the effort taken for setting up for in-ovo time lapse imaging is not insignificant [160]. Other researchers have also noted the opacity of the eggshell [105] and light-scattering properties of the egg yolk [159] to be imaging barriers when it comes to real-time observations. In contrast, PDMS models are able to offer better visibility as the embryo develops in a shell-less, optically clear environment, increasing compatibility for time-lapse imaging experiments [9, 105, 159]. The soft, transparent PDMS shell also not only enables visibility from the top-down view, but also from the sides and bottom, enabling multi-directional observations. Existing studies have well supported this idea of exploiting the PDMS eggshell for optically enhanced imaging research [9, 105, 161]. Last but not least, unlike traditional static in-ovo and ex-ovo systems, the PDMS model adopted in this study is also able to support the phenomenon of egg-turning, which plays a significant role in aiding the absorption of nutrients from the albumen, to the embryo [92].

Lastly, within the scope and purpose of this project, limitations of this research due to methodological problems should also be addressed. This is in particular to the sample size required for statistical measurements and logistical challenges faced for the duration of the study. Acquiring a sizeable sample count (5 or more) for each group on Day 18 proved to be a challenge, as it is established that viability in ex-ovo models is typically low, especially during the second week of incubation. Due to the period allocated to this study (one year), viability assessments only began in the second half of the year, with the first half optimizing the ex-ovo model in terms of fabrication, supplement parameters, and establishing the foundation for

cultivation early evaluation techniques. This can be alleviated by increasing the supply of fertilized eggs weekly to bolster sample counts necessary for analysis. However, due to external circumstances, the local supplier can only provide up to thirty to forty eggs per week, with the yield count of actual fertilized eggs with embryos to be about 25% after the initial three days of incubation, rendering the total number of embryos available per week not to be ideal. Notwithstanding rainy seasons or general supply issues that would affect the total count of fertilized eggs of this particular domestic breed available, it would undoubtedly hinder the progress of this experiment, primarily due to a time constraint. Therefore, naturally, the consensus would be that a greater sample size would enable the results to be generalized to a larger population, thus building a more robust case study and significance for the attained findings. The notion would then be to allocate ample logistical bandwidth in supply arrangements beforehand to achieve minimal study disruption. This would benefit future work based on this study's findings, facilitating greater identification of significant relationships in the data and formulating meaningful insights based on the initial trajectory of this study.

Summary

To summarize, several key insights have been formulated to rationalize the characteristic findings attained in this experiment:

1) The absence of an external calcium supply elicited a negative effect on the chicken embryo, conspicuous by the presence of feather abnormalities and overall poor viability compared to the other test groups.
2) External calcium supplementation was mainly beneficial to the chicken embryos, enhancing weight loss percentages, viability rates, whole-body length, and bone density volume in some specific cases.
3) The timing and quantity of external calcium supply are highly influential on all aspects of embryo growth and bone development.
    a. It was suggested that higher quantities of calcium as compared to lower quantities might perhaps be more substantial in producing adverse effects
    b. The distinction in findings between Day 3 and Day 10 groups might also possibly be attributed to the bioavailability of calcium presented to the embryos at each specific embryonic timepoint.

The inter-relationship between calcium quantity (50mg, 100mg), period of application (Day 3, 10), and the varying aspects of the study (embryonic development, weight loss, viability, morphometrics, bone density volume) are not easily characterized. This is because the effects of external calcium supply typically overlap one another, making it difficult to isolate any one aspect of the chicken embryo model itself. As such, a table has been generated in order to provide a comprehensive overview of the different groups and the results attained under different specific scenarios. This is useful for facilitating experimental goals, depending on the characteristics involved and the desired outcome of the experiment.

Legend
ED: Normal (✓), Abnormal (X)
WLP: >1x of Control (✓), <1x of Control (X)
VA: 1.5x (✓), 2x (✓✓), 3x, (✓✓✓)
MI: >1x of Control (✓), <1x of Control (-)
BVDA: >1x of Control (✓), <1x of Control (X)

	Embryonic Development	% Weight Loss Progression	Viability Assessment	Morphometrics Indicator	Bone Volume Density Analysis
Control Group	X	-	-	-	-
Calcium Group (D3, 50mg)	✓	✓	✓✓✓	✓	✓
Calcium Group (D3, 100mg)	✓	✓	✓	-	X
Calcium Group (D10, 50mg)	✓	X	✓✓	✓	X
Calcium Group (D10, 100mg)	X	X	✓	-	✓

Figure 23*: A summarized table comprising of the findings attained in this experiment. Each test group is graded accordingly based on the outcome for each specific characteristic method. The parameters for each grading criteria are as follow: 1) Embryonic Development: Normal (✓), Abnormal (X). 2) Weight Loss Progression: >1x of Control (✓), <1x of Control (X). 3) Viability Assessment: 1.5x of Control (✓), 2x (✓✓), 3x (✓✓✓). 4) Morphometrics Indicator: with significance >1x of Control (✓), <1x (-). 5) Bone Volume Density: >1x of Control (✓), <1x of Control (X). Amongst the test groups, the (D3, 50mg) Calcium groups yielded the overall best results in all selected aspects of the experiment.*

The summarized table above provides a clear outline of which parameters would be more suitable for different experimental studies. At first glance, it can be observed that although a particular test group (D10, 50mg) might excel in one category (viability), it might not present similar satisfactory results in other categories (bone volume density). It is then evident that the (D3, 50mg) Calcium group is the best performing category, especially within the viability rate. In future work, this sets good precedence in optimizing the calcium supplementation parameters for the ex-ovo PDMS transparent eggshell model. This is crucial as researchers

have long found ways to optimize avian embryo ex-ovo models to prolong survivability and sustainability for extensive research [25, 26, 33, 36].

Given that ex-ovo models are often utilized not only for early-stage experimental studies [10, 27] but also for late-stage [11, 15] or post-hatching [11, 16], there is significance in optimizing the PDMS ex-ovo model in this study to consider it as an alternative to other ex-ovo systems for chicken embryo research. Furthermore, the complete three-dimensional optical transparency and ease of embryonic accessibility of the model would be a valuable tool for developmental biology, taking advantage of various imaging modalities. Last but not least, as highlighted in the literature review, the PDMS ex-ovo system is biomimetic, economic, and sustainable, being able to support egg turning and has robust features that contribute to overall model sustainability [27].

Future Work

Future work is to be conducted to explore the potential applications and scalability of this transparent PDMS ex-ovo model. However, the research presented in this thesis has addressed some of the key topics regarding calcium effects and the optimization of this model. Hence, these give the direction to develop other potential areas of interest for future work, which will be further discussed below:

1. The first is utilizing different calcium supply sources or other supplements to evaluate this PDMS ex-ovo model. In the past, numerous types of calcium derivatives such as calcium carbonate, calcium lactate, calcium chloride, and even calcium-related nanoparticles have been explored using conventional avian ex-ovo systems [36, 40, 44, 162]. In addition, other supplements such as exogenous Vitamin D and K have also been researched [96]. Further work may include utilizing the suggestions above to determine if any is a feasible option for ex-ovo cultivation using this system. These alternatives may potentially be applied in further optimizing the PDMS model concerning long-term viability and sustainability.

2. The second will be to consider the introduction of oxygen aeration as an additional or standalone parameter when assessing characteristic elements of this ex-ovo model. Due to the nature of existing ex-ovo model designs, embryonic respiration may be affected, as the eggshell facilitates the gaseous exchange [36]. Past research has shown that embryonic survivability rates have improved with the aid of an external oxygen supply [25, 36, 40]. Since optimization methods around avian embryos' ex-ovo systems usually require a multi-faceted approach, it would be favorable to incorporate this aspect into future experiments for evaluation.

3. The third is the investigative possibility of the calcium absorption mechanism, including signaling pathways, especially for Day 3. Previously, it has been demonstrated that the deposition of calcium supplementation at the bottom of ex-ovo systems successfully enhanced the viability of chicken embryos [36, 40]. Consequently, the results attained in this

study were also congruent. As most studies often dedicate research to examining the involvement of calcium absorption via CAM on Day 10 onwards [96, 119], one possible research direction could be directed towards providing some form of clarity for calcium absorption via the bottom of the eggshell, especially since Day 3 supplementation results presented similar or better viability rates when compared to Day 10.

4. The fourth would be the usage of imaging modalities and exploring alternative disciplines to exploit the optical transparency features and embryonic accessibility of this PDMS ex-ovo model. Dr. Ishak formerly noted and discussed the possibility of accelerating the adoption of this ex-ovo model, particularly for imaging applications and education purposes [27]. This can likely be extended to advanced bioimaging techniques, such as photoacoustic imaging [163], high-frequency ultrasound [164], high field magnetic resonance imaging [165], and also fluorescence microscopy [113]. For example, Sharma et al. demonstrated using a similar PDMS ex-ovo model to conduct photoacoustic imaging studies, noting that the transparent eggshell was vital as the calcium eggshell often resulted in high signal attenuation [163]. Another study by Lai et al. also proposed adopting an "egg on a chip" plan using a cuboid PDMS ex-ovo model, citing advantages in optical transparencies and facilitating in-vivo 3D fluorescent imaging [9]. This study could serve as an avenue for prospective avenues in this forefront.

5. The fifth consideration will be to determine and assess the distribution and availability of the calcium in the experimental model. This will be necessary in order to affirm and supplement the findings that calcium uptake is quantity and time-dependent during the chicken embryo developmental phases. In order to more accurately evaluate calcium biodistribution, one must first measure the calcium contents of the supplement used, which in this case is eggshell powder. Composition analysis may be conducted to determine the mineral contents of the eggshell powder, as a comparison against literature data [149, 150] to determine

a baseline for bioavailability assessment. Once that is established, one may then estimate the calcium absorption via two possible means: the first proposed method is to carry out radiolabelling of the eggshell calcium supplement utilized in the experiment, which would enable the tracing of radioisotope distribution within the embryo as a function of embryonic development. Tuan demonstrated the effectiveness of this method by radiolabelling eggshell pieces to be used as calcium repletion for the chicken embryos, successfully tracing the mobilization of eggshell calcium throughout the embryonic system [43]. The second alternative would be to quantify the amount of calcium ion present in the amniotic fluid of the chicken embryo. A study by Vargas et al. discovered that a notable increase in amniotic calcium fluid content was distinguished five days later after 45S5 Bioglass-derived glass-ceramic scaffolds were implanted on the CAM of shell-less chicken embryos [166]. In addition, alongside with findings of enhanced bone mineralization in embryo groups implanted with this scaffold, it was deduced that the calcium release from the biomaterial might have been responsible for the two phenomenons observed above [166]. As such, following the methods suggested above, the differences in calcium bioavailability and distribution between the control and different embryonic groups may then possibly be investigated.

Conclusion

In essence, this research aims to optimize a transparent biomimetic PDMS eggshell for ex-ovo chicken embryo models by investigating the effects of eggshell powder as an external calcium supplementation. This was achieved based on a quantitative and qualitative approach in response to the chicken embryo's overall growth and bone development by varying calcium quantity and period of application. As a result, calcium supplement parameters and timepoint were successfully optimized to enhance percentage weight loss progression, bone volume density, viability rates, and whole-body sizes. Furthermore, chicken embryos supplemented with a lower quantity of calcium (50mg) at an earlier embryonic time point (Day 3) produced the best results.

The findings indicate that the scarcity of an extraembryonic calcium source appears to realize an adverse effect concerning a greatly diminished survivability rate, as illustrated by the Control group. In addition, the two to three times increase in survivability rates for embryos supplied with eggshell powder suggests that any external supplementation is incredibly beneficial to the cause. Lastly, this study clearly illustrates that the dosage and period of application are essential criteria for consideration when a lower quantity was perceived to generate a more favorable response, citing the possibility of an ‘optimal window’ to sustain necessary growth conditions. Future studies could address current implications, such as the type of calcium utilized or introducing new parameters such as oxygen aeration. In addition, more research is required to investigate the underlying calcium absorption mechanism and signaling pathways for calcium deposition on embryonic day three. Overall, the feasibility of this PDMS model as an ex-ovo system potentially renders it an effective tool for developmental biology studies and possibly supporting imaging modalities to a greater extent. This makes it an economical and sustainable solution suitable for late-stage research operations.

References

1. Rashidi, H. and V. Sottile, *The chick embryo: hatching a model for contemporary biomedical research.* BioEssays, 2009. **31**(4): p. 459-465.
2. Robinson, N.B., et al., *The current state of animal models in research: A review.* Int J Surg, 2019. **72**: p. 9-13.
3. Gerdts, V., et al., *Large animal models for vaccine development and testing.* Ilar j, 2015. **56**(1): p. 53-62.
4. Stern, C.D., *The Chick: A Great Model System Becomes Even Greater.* Developmental Cell, 2005. **8**(1): p. 9-17.
5. Stern, C.D., *The chick embryo--past, present and future as a model system in developmental biology.* Mech Dev, 2004. **121**(9): p. 1011-3.
6. Bednarczyk, M., et al., *Chicken embryo as a model in epigenetic research.* Poultry Science, 2021. **100**(7): p. 101164.
7. Fauzia, E., et al., *Chick Embryo: A Preclinical Model for Understanding Ischemia-Reperfusion Mechanism.* Frontiers in Pharmacology, 2018. **9**(1034).
8. Wilson, H.R., *Physiological requirements of the developing embryo: temperature and turning*. 1990, Butterworth (Publishers) Ltd: Kent. p. 145-156.
9. Lai, Y. and J. Liu, *Transparent soft PDMS eggshell.* Science China Technological Sciences, 2015. **58**(2): p. 273-283.
10. Kulesa, P.M. and S.E. Fraser, *In ovo time-lapse analysis of chick hindbrain neural crest cell migration shows cell interactions during migration to the branchial arches.* Development, 2000. **127**(6): p. 1161-72.
11. Coleman, C.M., *Chicken embryo as a model for regenerative medicine.* Birth Defects Research Part C: Embryo Today: Reviews, 2008. **84**(3): p. 245-256.
12. Goodpasture, E.W., A.M. Woodruff, and G.J. Buddingh, *The Cultivation of Vaccine and Other Viruses in the Chorio-Allantoic Membrane of Chick Embryos.* Science, 1931. **74**(1919): p. 371-372.
13. Ribatti, D., *The chick embryo chorioallantoic membrane (CAM). A multifaceted experimental model.* Mechanisms of Development, 2016. **141**: p. 70-77.
14. MURPHY, J.B., *TRANSPLANTABILITY OF MALIGNANT TUMORS TO THE EMBRYOS OF A FOREIGN SPECIES.* Journal of the American Medical Association, 1912. **LIX**(11): p. 874-875.
15. Kain, K.H., et al., *The chick embryo as an expanding experimental model for cancer and cardiovascular research.* Developmental Dynamics, 2014. **243**(2): p. 216-228.
16. Mozdziak, P.E., et al., *Development of transgenic chickens expressing bacterial beta-galactosidase.* Dev Dyn, 2003. **226**(3): p. 439-45.
17. Guerrero-Bosagna, C., et al., *Transgenerational epigenetic inheritance in birds.* Environ Epigenet, 2018. **4**(2): p. dvy008.
18. Jourdeuil, K.A., C.L. Hammer, and T.A. Franz-Odendaal, *A comparative analysis of chick culturing methods on skeletogenesis.* Anat Rec (Hoboken), 2015. **298**(5): p. 810-9.
19. Fujita, Y., et al. *Influence of surrogate eggshell environment on development of cultured chicken embryos*. 2007.
20. Borwompinyo, S., et al., *Culture of chicken embryos in surrogate eggshells.* Poultry Science, 2005. **84**(9): p. 1477-1482.
21. Andacht, T., W. Hu, and R. Ivarie, *Rapid and improved method for windowing eggs accessing the stage X chicken embryo.* Molecular Reproduction and Development, 2004. **69**(1): p. 31-34.

22. Speksnijder, G. and R. Ivarie, *A modified method of shell windowing for producing somatic or germline chimeras in fertilized chicken eggs.* Poult Sci, 2000. **79**(10): p. 1430-3.
23. Farzaneh, M., et al., *The method of chicken whole embryo culture using the eggshell windowing, surrogate eggshell and ex ovo culture system.* British Poultry Science, 2018. **59**(2): p. 240-244.
24. New, D., *A New Technique for the Cultivation of the Chick Embryo in vitro.* Development, 1955. **3**: p. 326-331.
25. Tahara, Y. and K. Obara, *A Novel Shell-less Culture System for Chick Embryos Using a Plastic Film as Culture Vessels.* The Journal of Poultry Science, 2014. **51**(3): p. 307-312.
26. Yalcin, H.C., et al., *An ex-ovo chicken embryo culture system suitable for imaging and microsurgery applications.* Journal of visualized experiments : JoVE, 2010(44): p. 2154.
27. Ishak, N., et al., *Bioengineered three-dimensional transparent eggshell as a chicken embryo experimentation platform for biomedical research.* Engineering Reports, 2020. **2**(1): p. e12092.
28. Narushin, V.G. and M.N. Romanov, *Egg physical characteristics and hatchability.* World's Poultry Science Journal, 2002. **58**(3): p. 297-303.
29. Stoddard, M.C., et al., *Avian egg shape: Form, function, and evolution.* Science, 2017. **356**(6344): p. 1249-1254.
30. King'Ori, A., *Review of the factors that influence egg fertility and hatchability in poultry.* International Journal of poultry science, 2011. **10**(6): p. 483-492.
31. Darnell, D.K. and G.C. Schoenwolf, *The Chick Embryo as a Model System for Analyzing Mechanisms of Development*, in *Developmental Biology Protocols: Volume I*, J.M. Walker, R.S. Tuan, and C.W. Lo, Editors. 2000, Humana Press: Totowa, NJ. p. 25-29.
32. Auerbach, R., et al., *A simple procedure for the long-term cultivation of chicken embryos.* Developmental Biology, 1974. **41**(2): p. 391-394.
33. Cloney, K. and T.A. Franz-Odendaal, *Optimized ex-ovo culturing of chick embryos to advanced stages of development.* Journal of visualized experiments : JoVE, 2015(95): p. 52129-52129.
34. Deeming, D.C., *The Role of Egg Turning during Incubation.* Avian Biology Research, 2009. **2**(1-2): p. 67-71.
35. Deeming, D.C., *Characteristics of unturned eggs: Critical period, retarded embryonic growth and poor albumen utilisation.* British Poultry Science, 1989. **30**(2): p. 239-249.
36. Kamihira, M., et al., *Improved hatching for in vitro quail embryo culture using surrogate eggshell and artificial vessel.* Development, Growth & Differentiation, 1998. **40**(4): p. 449-455.
37. Miyahara, T., R.E. Akins, and R.S. Tuan, *Alterations in cellular calcium handling as a result of systemic calcium deficiency in the developing chick embryo: II. Ventricular myocytes.* J Cell Physiol, 1992. **153**(3): p. 636-44.
38. Halgrain, M., et al., *Eggshell decalcification and skeletal mineralization during chicken embryonic development: defining candidate genes in the chorioallantoic membrane.* Poultry Science, 2022. **101**(2): p. 101622.
39. Ono, T. and R.S. Tuan, *Effect of experimentally induced calcium deficiency on development, metabolism and liver morphogenesis of the chick embryo.* J Embryol Exp Morphol, 1986. **92**: p. 207-22.

40. Tahara, Y., K. Obara, and M. Kamihira, *Calcium carbonate supplementation to chorioallantoic membranes improves hatchability in shell-less chick embryo culture.* Journal of Bioscience and Bioengineering, 2021. **131**(3): p. 314-319.
41. Tuan, R.S., *Calcium transport and related functions in the chorioallantoic membrane of cultured shell-less chick embryos.* Developmental Biology, 1980. **74**(1): p. 196-204.
42. Tuan, R.S. and M.H. Lynch, *Effect of experimentally induced calcium deficiency on the developmental expression of collagen types in chick embryonic skeleton.* Dev Biol, 1983. **100**(2): p. 374-86.
43. Tuan, R.S., *Supplemented eggshell restores calcium transport in chorioallantoic membrane of cultured shell-less chick embryos.* Journal of Embryology and Experimental Morphology, 1983. **74**(1): p. 119-131.
44. Matuszewski, A., et al., *Calcium Carbonate Nanoparticles—Toxicity and Effect of In Ovo Inoculation on Chicken Embryo Development, Broiler Performance and Bone Status.* Animals, 2021. **11**(4): p. 932.
45. SEABRA, R. and N. BHOGAL, *In Vivo Research Using Early Life Stage Models.* In Vivo, 2010. **24**(4): p. 457-462.
46. Nowak-Sliwinska, P., T. Segura, and M.L. Iruela-Arispe, *The chicken chorioallantoic membrane model in biology, medicine and bioengineering.* Angiogenesis, 2014. **17**(4): p. 779-804.
47. Naito, M., *Embryo Manipulation in Chickens.* The Journal of Poultry Science, 2015. **52**(1): p. 7-14.
48. Hamburger, V. and H.L. Hamilton, *A series of normal stages in the development of the chick embryo.* Journal of Morphology, 1951. **88**(1): p. 49-92.
49. Mast, J. and B.M. Goddeeris, *Development of immunocompetence of broiler chickens.* Vet Immunol Immunopathol, 1999. **70**(3-4): p. 245-56.
50. Seto, F., *Early Development of the Avian Immune System1.* Poultry Science, 1981. **60**(9): p. 1981-1995.
51. Perry, M.M., *A complete culture system for the chick embryo.* Nature, 1988. **331**(6151): p. 70-72.
52. Psychoyos, D. and R. Finnell, *Method for Culture of Early Chick Embryos ex vivo (New Culture).* Journal of visualized experiments : JoVE, 2008(20): p. 903.
53. Endo, Y., *Chick embryo culture and electroporation.* Current protocols in cell biology, 2012. **Chapter 19**: p. Unit19.15-Unit19.15.
54. Korn, M.J. and K.S. Cramer, *Windowing chicken eggs for developmental studies.* J Vis Exp, 2007(8): p. 306.
55. El-Ghali, N., et al., *New methods for chicken embryo manipulations.* Microscopy Research and Technique, 2010. **73**(1): p. 58-66.
56. Jessl, L., J. Scheider, and J. Oehlmann, *The domestic fowl (Gallus gallus domesticus) embryo as an alternative for mammalian experiments – Validation of a test method for the detection of endocrine disrupting chemicals.* Chemosphere, 2018. **196**: p. 502-513.
57. Dunn, B.E., *Technique for Shell-Less Culture of the 72-Hour Avian Embryo.* Poultry Science, 1974. **53**(1): p. 409-412.
58. Vince, M.A., *Some Environmental Effects on the Activity and Development of the Avian Embryo*, in *Studies on the Development of Behavior and the Nervous System*, G. Gottlieb, Editor. 1973, Elsevier. p. 285-323.
59. Doty, M., *Hamburger-Hamilton Staging Series (1951)*, in *Embryo Project Encyclopedia (2011-06-10)*. 1951, Arizona State University. School of Life Sciences. Center for Biology and Society. Embryo Project Encyclopedia.

60. Spratt Jr., N.T., *Location of organ-specific regions and their relationship to the development of the primitive streak in the early chick blastoderm.* Journal of Experimental Zoology, 1942. **89**(1): p. 69-101.
61. El-Ashram, M.M. *Egg Turning During Incubation – a review by Mohamed Mostafa El-Ashram*. 2020 [cited 2021 08 April]; Available from: https://www.emtech-systems.com/technical-talk/egg-turning-during-incubation-a-review-by-mohamed-mostafa-el-ashram/.
62. Cutchin, H., et al., *Embryonic development when eggs are turned different angles during incubation.* Journal of Applied Poultry Research - J APPL POULTRY RES, 2009. **18**: p. 447-451.
63. Boleli, I., et al., *Poultry Egg Incubation: Integrating and Optimizing Production Efficiency.* Revista Brasileira de Ciência Avícola, 2016. **18**: p. 1-16.
64. Tazawa, H., *Adverse effect of failure to turn the avian egg on the embryo oxygen exchange.* Respiration Physiology, 1980. **41**(2): p. 137-142.
65. Eycleshymer, A.C., *SOME OBSERVATIONS AND EXPERIMENTS ON THE NATURAL AND ARTIFICIAL INCUBATION OF THE EGG OF THE COMMON FOWL.* The Biological Bulletin, 1907. **12**(6): p. 360-374.
66. Romanoff, A.L., *The avian embryo : structural and functional development*. 1960: New York (N.Y.) : Macmillan.
67. Freeman, B.M., *Development of the avian embryo; a behavioural and physiological study [by] B. M. Freeman and Margaret A. Vince*, ed. M.A. Vince. 1974, London, New York: Chapman and Hall; Wiley.
68. New, D., *A Critical Period for the Turning of Hens' Eggs.* Development, 1957. **5**: p. 293-299.
69. Elibol, O. and J. Brake, *Identification of critical periods for turning broiler hatching eggs during incubation.* British Poultry Science, 2004. **45**(5): p. 631-637.
70. Tyrrell, D.A., et al., *A new count of allontoic cells of the 10-day chick embryo.* Proc Soc Exp Biol Med, 1954. **86**(3): p. 594-8.
71. Tullett, S.G. and D.C. Deeming, *Failure to turn eggs during incubation: effects on embryo weight, development of the chorioallantois and absorption of albumen.* Br Poult Sci, 1987. **28**(2): p. 239-49.
72. Betz, T.W., *The effects of embryonic pars distalis grafts and albumen on the growth of chick embryos.* J Embryol Exp Morphol, 1968. **20**(3): p. 431-6.
73. da S. Oliveira, G., et al., *Effects of different egg turning frequencies on incubation efficiency parameters.* Poultry Science, 2020. **99**(9): p. 4417-4420.
74. *Brinsea Handbook*. 2016.
75. Landauer, W. *Hatchability of Chicken Eggs as Influenced by Environment and Heredity, The*. 1961.
76. Elibol, O. and J. Braket, *Effect of frequency of turning from three to eleven days of incubation on hatchability of broiler hatching eggs.* Poult Sci, 2003. **82**(3): p. 357-9.
77. French, N.A., *Modeling incubation temperature: the effects of incubator design, embryonic development, and egg size.* Poultry Science, 1997. **76**(1): p. 124-133.
78. Funk, E.M. and J. Forward, *The Relation of Angle of Turning and Position of the Egg to Hatchability of Chicken Eggs.* Poultry Science, 1960. **39**(3): p. 784-785.
79. Elibol, O. and J. Brake, *Effect of Egg Turning Angle and Frequency During Incubation on Hatchability and Incidence of Unhatched Broiler Embryos with Head in the Small End of the Egg1 1The use of trade names in this publication does not imply endorsement of the products mentioned nor criticism of similar products not mentioned.* Poultry Science, 2006. **85**(8): p. 1433-1437.

80. Tona, K., et al., *Effects of turning duration during incubation on embryo growth, utilization of albumen, and stress regulation.* Poult Sci, 2005. **84**(2): p. 315-20.
81. Kulesa, P.M., et al., *In ovo live imaging of avian embryos.* Cold Spring Harbor protocols, 2010. **2010**(6): p. pdb.prot5446-pdb.prot5446.
82. Salter, D.W., et al., *Gene insertion into the chicken germ line by retroviruses.* Poult Sci, 1986. **65**(8): p. 1445-58.
83. Liu, C., et al., *Culture of chicken embryo in interspecific surrogate egg white.* Poultry science, 2012. **91**: p. 2866-71.
84. van de Lavoir, M.-C., et al., *Germline transmission of genetically modified primordial germ cells.* Nature, 2006. **441**(7094): p. 766-769.
85. Nakamura, H., et al., *Gain- and loss-of-function in chick embryos by electroporation.* Mechanisms of Development, 2004. **121**(9): p. 1137-1143.
86. Li, Z.D., et al., *Production of duck-chicken chimeras by transferring early blastodermal cells.* Poult Sci, 2002. **81**(9): p. 1360-4.
87. Cuperus, T., et al., *Protective effect of in ovo treatment with the chicken cathelicidin analog D-CATH-2 against avian pathogenic E. coli.* Scientific Reports, 2016. **6**(1): p. 26622.
88. Lee, H.J., H.C. Lee, and J.Y. Han, *Germline Modification and Engineering in Avian Species.* Molecules and cells, 2015. **38**(9): p. 743-749.
89. Bosselman, R.A., et al., *Replication-defective vectors of reticuloendotheliosis virus transduce exogenous genes into somatic stem cells of the unincubated chicken embryo.* Journal of virology, 1989. **63**(6): p. 2680-2689.
90. Nguyen, M., Y. Shing, and J. Folkman, *Quantitation of angiogenesis and antiangiogenesis in the chick embryo chorioallantoic membrane.* Microvasc Res, 1994. **47**(1): p. 31-40.
91. Uchibayashi, T., et al., *Responses of tumour cell lines implanted onto the chorioallantoic membrane of chick embryo to anticancer agents in combination with hyperthermia.* Urol Res, 1992. **20**(3): p. 237-9.
92. Rowlett, K. and K. Simkiss, *Explanted embryo culture: In vitro and in ovo techniques for domestic fowl.* British Poultry Science, 1987. **28**(1): p. 91-101.
93. Fineman, R.M. and G.C. Schoenwolf, *Animal model: dysmorphogenesis and death in a chicken embryo model.* Am J Med Genet, 1987. **27**(3): p. 543-52.
94. Fisher, M. and G.C. Schoenwolf, *The use of early chick embryos in experimental embryology and teratology: Improvements in standard procedures.* Teratology, 1983. **27**(1): p. 65-72.
95. Naito, M., K. Nirasawa, and T. Oishi, *Development in culture of the chick embryo from fertilized ovum to hatching.* Journal of Experimental Zoology, 1990. **254**(3): p. 322-326.
96. Tuan, R.S. and T. Ono, *Regulation of extraembryonic calcium mobilization by the developing chick embryo.* Journal of Embryology and Experimental Morphology, 1986. **97**(1): p. 63-74.
97. Dunn, B.E., T.P. Fitzharris, and B.D. Barnett, *Effects of varying chamber construction and embryo pre-incubation age on survival and growth of chick embryos in shell-less culture.* Anat Rec, 1981. **199**(1): p. 33-43.
98. Gong, X. and S. He, *Highly Durable Superhydrophobic Polydimethylsiloxane/Silica Nanocomposite Surfaces with Good Self-Cleaning Ability.* ACS omega, 2020. **5**(8): p. 4100-4108.
99. Cho, C.-F., et al., *Evaluation of nanoparticle uptake in tumors in real time using intravital imaging.* Journal of visualized experiments : JoVE, 2011(52): p. 2808.

100. Dohle, D., et al., *Chick ex ovo Culture and ex ovo CAM Assay: How it Really Works.* Journal of visualized experiments : JoVE, 2009. **32**.
101. Oppitz, M., et al., *Non-malignant migration of B16 mouse melanoma cells in the neural crest and invasive growth in the eye cup of the chick embryo.* Melanoma Res, 2007. **17**(1): p. 17-30.
102. Höckel, M., J. Sasse, and J.H. Wissler, *Purified monocyte-derived angiogenic substance (angiotropin) stimulates migration, phenotypic changes, and "tube formation" but not proliferation of capillary endothelial cells in vitro.* J Cell Physiol, 1987. **133**(1): p. 1-13.
103. Kistler, H.B., Jr and J.H. LaVail, *Penetration of horseradish peroxidase into the optic nerve after vitreal or vascular injections in the developing chick.* Investigative Ophthalmology & Visual Science, 1981. **20**(6): p. 705-716.
104. Scadding, S.R., *How to Culture Chicken Embryos in Plastic Wrap Suspension.* The American Biology Teacher, 1985. **47**(2): p. 107-108.
105. Huang, W., F. Arai, and T. Kawahara, *Egg-in-Cube: Design and Fabrication of a Novel Artificial Eggshell with Functionalized Surface.* PLOS ONE, 2015. **10**(3): p. e0118624.
106. Philip, R.B., et al., *Multidisciplinary Inquiry-Based Investigation Learning Using an Ex Ovo Chicken Culture Platform: Role of Vitamin A on Embryonic Morphogenesis.* The American Biology Teacher, 2012. **74**(9): p. 636-643.
107. Bastarrachea, L., S. Dhawan, and S. Sablani, *Engineering Properties of Polymeric-Based Antimicrobial Films for Food Packaging: A Review.* Food Engineering Reviews, 2011. **3**: p. 79-93.
108. contributors, W. *Polydimethylsiloxane. In Wikipedia, The Free Encyclopedia*. 2021 [cited 2021 28 July]; Available from: https://en.wikipedia.org/w/index.php?title=Polydimethylsiloxane&oldid=1031244721 .
109. Sia, S.K. and G.M. Whitesides, *Microfluidic devices fabricated in Poly(dimethylsiloxane) for biological studies.* ELECTROPHORESIS, 2003. **24**(21): p. 3563-3576.
110. Gale, B., et al., *Low-Cost MEMS Technologies*. 2008. p. 341-378.
111. Bélanger, M.C. and Y. Marois, *Hemocompatibility, biocompatibility, inflammatory and in vivo studies of primary reference materials low-density polyethylene and polydimethylsiloxane: a review.* J Biomed Mater Res, 2001. **58**(5): p. 467-77.
112. El-Ali, J., P.K. Sorger, and K.F. Jensen, *Cells on chips.* Nature, 2006. **442**(7101): p. 403-411.
113. D'Alba, L., et al., *Antimicrobial properties of a nanostructured eggshell from a compost-nesting bird.* Journal of Experimental Biology, 2014. **217**(7): p. 1116-1121.
114. Solomon, S.E., *The eggshell: strength, structure and function.* British Poultry Science, 2010. **51**(sup1): p. 52-59.
115. Hincke, M., et al., *8 - The eggshell: structure and protective function*, in *Improving the Safety and Quality of Eggs and Egg Products*, Y. Nys, M. Bain, and F. Van Immerseel, Editors. 2011, Woodhead Publishing. p. 151-182.
116. SIMKISS, K., *CALCIUM METABOLISM AND AVIAN REPRODUCTION.* Biological Reviews, 1961. **36**(3): p. 321-359.
117. Romanoff, A.L. and A.J. Romanoff, *Biochemistry of the Avian Embryo: A Quantitative Analysis of Prenatal Development*. 1967: Interscience Publishers.
118. Packard, M.J. and G.C. Packard, *Comparative aspects of calcium metabolism in embryonic reptiles and birds*, in *Respiration and metabolism of embryonic vertebrates: Satellite Symposium of the 29th International Congress of Physiological*

Sciences, Sydney, Australia, 1983, R.S. Seymour, Editor. 1984, Springer Netherlands: Dordrecht. p. 155-179.
119. Tuan, R.S. and W.A. Scott, *Calcium-binding protein of chorioallantoic membrane: identification and development expression.* Proceedings of the National Academy of Sciences of the United States of America, 1977. **74**(5): p. 1946-1949.
120. Tuan, R.S., W.A. Scott, and Z.A. Cohn, *Purification and characterization of calcium-binding protein from chick chorioallantoic membrane.* Journal of Biological Chemistry, 1978. **253**(4): p. 1011-1016.
121. Kamihira, M., et al., *High-level expression of single-chain Fv-Fc fusion protein in serum and egg white of genetically manipulated chickens by using a retroviral vector.* Journal of virology, 2005. **79**(17): p. 10864-10874.
122. Rovenský, J., et al., *Eggshell calcium in the prevention and treatment of osteoporosis.* Int J Clin Pharmacol Res, 2003. **23**(2-3): p. 83-92.
123. Waheed, M., et al., *Eggshell calcium: A cheap alternative to expensive supplements.* Trends in Food Science & Technology, 2019. **91**: p. 219-230.
124. M.M. Cordeiro, C. and M. T. Hincke, *Recent Patents on Eggshell: Shell and Membrane Applications.* Recent Patents on Food, Nutrition & Agriculture, 2011. **3**(1): p. 1-8.
125. Hincke, M., et al., *The eggshell: structure, composition and mineralization.* Frontiers in bioscience : a journal and virtual library, 2012. **17**: p. 1266-80.
126. Frear, G.L. and J. Johnston, *THE SOLUBILITY OF CALCIUM CARBONATE (CALCITE) IN CERTAIN AQUEOUS SOLUTIONS AT 25°1.* Journal of the American Chemical Society, 1929. **51**(7): p. 2082-2093.
127. Quina, M.J., M.A.R. Soares, and R. Quinta-Ferreira, *Applications of industrial eggshell as a valuable anthropogenic resource.* Resources, Conservation and Recycling, 2017. **123**: p. 176-186.
128. Richards, M.P., *Trace mineral metabolism in the avian embryo.* Poult Sci, 1997. **76**(1): p. 152-64.
129. Romanoff, A.L.R.A.J., *The avian egg.* 1949.
130. Hamilton, W.J., *Lillie's Development of the Chick—an Introduction to Embryology.* Journal of Anatomy, 1953. **87**(Pt 2): p. 217-217.
131. Borwompinyo, S., et al., *Culture of chicken embryos in surrogate eggshells.* Poult Sci, 2005. **84**(9): p. 1477-82.
132. Torres, C.A. and D.R. Korver, *Influences of trace mineral nutrition and maternal flock age on broiler embryo bone development.* Poultry Science, 2018. **97**(8): p. 2996-3003.
133. Leeson, S. and T. Walsh, *Feathering in commercial poultry II. Factors influencing feather growth and feather loss.* World's Poultry Science Journal, 2004. **60**(1): p. 52-63.
134. Li, A., et al., *Calcium oscillations coordinate feather mesenchymal cell movement by SHH dependent modulation of gap junction networks.* Nature Communications, 2018. **9**(1): p. 5377.
135. Chang, K.-W., et al., *Emergence of differentially regulated pathways associated with the development of regional specificity in chicken skin.* BMC Genomics, 2015. **16**(1): p. 22.
136. Deeming, D.C., *Factors affecting hatchability during commercial incubation of Ostrich (Struthio camelus) eggs.* British Poultry Science, 1995. **36**(1): p. 51-65.
137. Tona, J., et al., *Relationship Between Broiler Breeder's Age and Egg Weight Loss and Embryonic Mortality During Incubation in Large-Scale Conditions.* The Journal of Applied Poultry Research, 2001. **10**.

138. Packard, M.J. and N.B. Clark, *Calcium regulation in the embryonic chick. III. Calcium and phosphate in serum and allantoic fluid of normal and shell-less embryos.* The Journal of experimental zoology. Supplement : published under auspices of the American Society of Zoologists and the Division of Comparative Physiology and Biochemistry, 1987. **1**: p. 25-32.
139. Richards, M.P., R.W. Rosebrough, and N.C. Steele, *Hepatic zinc, copper and iron of turkey embryos (Meleagris gallapavo) maintained in long-term, shell-less culture.* Comparative Biochemistry and Physiology Part A: Physiology, 1984. **78**(3): p. 525-531.
140. Scott, M., E. Holm, and R. Reynolds, *Studies on the Niacin, Riboflavin, Choline, Manganese and Zinc Requirements of Young Ringnecked Pheasants for Growth, Feathering and Prevention of Leg Disorders.* Poultry Science, 1959. **38**: p. 1344-1350.
141. Soetan, K., C. Olaiya, and O. Oyewole, *The importance of mineral elements for humans, domestic animals and plants: A review.* Afr J Food Sci, 2009. **4**.
142. Pass, D.A., *The pathology of the avian integument: a review.* Avian Pathol, 1989. **18**(1): p. 1-72.
143. de Matos, R., *Calcium metabolism in birds.* Vet Clin North Am Exot Anim Pract, 2008. **11**(1): p. 59-82, vi.
144. Arena, S., G. Renzone, and A. Scaloni, *A multi-approach peptidomic analysis of hen egg white reveals novel putative bioactive molecules.* Journal of Proteomics, 2020. **215**: p. 103646.
145. Willems, E., et al., *Importance of albumen during embryonic development in avian species, with emphasis on domestic chicken.* World's Poultry Science Journal, 2014. **70**(3): p. 503-518.
146. Sharif, M.K., M. Saleem, and K. Javed, *Chapter 15 - Food Materials Science in Egg Powder Industry*, in *Role of Materials Science in Food Bioengineering*, A.M. Grumezescu and A.M. Holban, Editors. 2018, Academic Press. p. 505-537.
147. Wolf, S.E., et al., *Strong stabilization of amorphous calcium carbonate emulsion by ovalbumin: gaining insight into the mechanism of 'polymer-induced liquid precursor' processes.* Journal of the American Chemical Society, 2011. **133**(32): p. 12642-12649.
148. Baggott, G.K., *Development of Extra-embryonic Membranes and Fluid Compartments.* Avian Biology Research, 2009. **2**(1-2): p. 21-26.
149. Szeleszczuk, Ł., et al., *Analysis of the changes in elemental composition of the chicken eggshell during the incubation period.* Journal of Animal and Plant Sciences, 2016. **26**: p. 583-587.
150. Schaafsma, A., et al., *Mineral, amino acid, and hormonal composition of chicken eggshell powder and the evaluation of its use in human nutrition.* Poultry Science, 2000. **79**(12): p. 1833-1838.
151. Szeleszczuk, Ł., et al., *In Vitro Dissolution of Calcium Carbonate from the Chicken Eggshell: A Study of Calcium Bioavailability.* International Journal of Food Properties, 2015. **18**(12): p. 2791-2799.
152. Schomann, T., et al., *Improved Method for Ex Ovo-Cultivation of Developing Chicken Embryos for Human Stem Cell Xenografts.* Stem Cells International, 2013. **2013**: p. 960958.
153. Tuan, R., *Supplemented eggshell restores calcium transport in chorioallantoic membrane of cultured shell-less chick embryos.* Journal of embryology and experimental morphology, 1983. **74**: p. 119-31.
154. Liu, C., et al., *Culture of chicken embryo in interspecific surrogate egg white.* Poult Sci, 2012. **91**(11): p. 2866-71.

155. Paudel, S., R. Sindelar, and M. Saha, *Calcium Signaling in Vertebrate Development and Its Role in Disease.* Int J Mol Sci, 2018. **19**(11).
156. Miyahara, T., R.E. Akins, and R.S. Tuan, *Alterations in cellular calcium handling as a result of systemic calcium deficiency in the developing chick embryo: II. Ventricular myocytes.* Journal of Cellular Physiology, 1992. **153**(3): p. 636-644.
157. De Pablo, F., et al., *Insulin-Like Growth Factor-I and Insulin as Growth and Differentiation Factors in Chicken Embryogenesis.* Poultry Science, 1991. **70**(8): p. 1790-1796.
158. De Pablo, F., et al., *IGF-I and the IGF-I receptor in development of nonmammalian vertebrates.* Molecular Reproduction and Development, 1993. **35**(4): p. 427-433.
159. El-Ghali, N., et al., *New methods for chicken embryo manipulations.* Microsc Res Tech, 2010. **73**(1): p. 58-66.
160. Ezin, M. and S. Fraser, *Chapter 11 Time‐Lapse Imaging of the Early Avian Embryo*, in *Methods in Cell Biology*. 2008, Academic Press. p. 211-236.
161. Sharma, A., et al., *High resolution, label-free photoacoustic imaging of live chicken embryo developing in bioengineered eggshell.* Journal of Biophotonics, 2020. **13**(4): p. e201960108.
162. Johnston, P.M. and C.L. Comar, *Distribution and Contribution of Calcium From the Albumen, Yolk and Shell to the Developing Chick Embryo.* American Journal of Physiology-Legacy Content, 1955. **183**(3): p. 365-370.
163. Sharma, A., et al., *Photoacoustic imaging of live chicken embryo at multiple developmental stages*. SPIE BiOS. Vol. 11240. 2020: SPIE.
164. Tan, G.X., et al., *3D Reconstruction of Chick Embryo Vascular Geometries Using Non-invasive High-Frequency Ultrasound for Computational Fluid Dynamics Studies.* Ann Biomed Eng, 2015. **43**(11): p. 2780-93.
165. Bain, M.M., et al., *Noninvasive monitoring of chick development in ovo using a 7T MRI system from day 12 of incubation through to hatching.* J Magn Reson Imaging, 2007. **26**(1): p. 198-201.
166. Vargas, G.E., et al., *Biocompatibility and bone mineralization potential of 45S5 Bioglass-derived glass-ceramic scaffolds in chick embryos.* Acta Biomater, 2009. **5**(1): p. 374-80.