Arrested embryonic development: a review of strategies to delay hatching in egg-laying reptiles

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Arrested embryonic development involves the downregulation or cessation of active cell division and metabolic activity, and the capability of an animal to arrest embryonic development results in temporal plasticity of the duration of embryonic period. Arrested embryonic development is an important reproductive strategy for egg-laying animals that provide no parental care after oviposition. In this review, we discuss each type of embryonic developmental arrest used by oviparous reptiles. Environmental pressures that might have directed the evolution of arrest are addressed and we present previously undiscussed environmentally dependent physiological processes that may occur in the egg to bring about arrest. Areas for future research are proposed to clarify how ecology affects the phenotype of developing embryos. We hypothesize that oviparous reptilian mothers are capable of providing their embryos with a level of phenotypic adaptation to local environmental conditions by incorporating maternal factors into the internal environment of the egg that result in different levels of developmental sensitivity to environmental conditions after they are laid.

Keywords: amniotic; arrested development; ecology; evolution; reproduction; reptile

1. INTRODUCTION

Evolution of the amniotic egg was paramount in eliminating the need for vertebrate animals to reproduce in water, and allowed independent embryonic existence in the absence of parental care [1]. The amniotic egg was a major evolutionary adaptation that facilitated terrestrial exploitation, because on land, eggs are exposed to a highly variable environment in comparison with the relatively stable aquatic system [2]. In order to deal with potentially adverse environmental conditions, several lineages evolved ovoviparity—the production of eggs that develop and hatch within the female's body, resulting in the birth of a relatively well-developed neonate [3], whereas those that retained oviparity (egg-laying) developed the ability to arrest embryonic development inside the egg until favourable conditions arise or return. Arrested embryonic development is an adaptation characterized by the downregulation or cessation of active cell division and metabolic activity of the embryo [4]. It is a reproductive strategy that is employed by many taxa including plants, insects and amniotic vertebrates, suggesting that it has evolved independently on numerous occasions [5]. This life-history trait confers a significant selective advantage because it allows embryos to respond to varying environmental conditions by altering their period of development [6].

Extant reptiles (squamates, crocodiles, turtles and the tuatara) possess the most diverse range of strategies to prolong the egg state, while in mammals and birds these mechanisms are less varied because of their higher level of parental care [4]. The incidence of developmental arrest decreases in mammalian species that provide more parental care, and bird embryos typically only retard development when parents are not tending to them [7–9]. The rich abundance of strategies to arrest development in reptiles enables embryos to withstand a changing incubation environment in a variety of ecological settings [10]. Most reptilian species undergo at least one period of developmental arrest while inside the amniotic egg, and in some cases both before and after oviposition [4,11], as summarized in figure 1.

Arrested embryonic development in mammalian species that provide parental input and care during embryonic development and after parturition has been reviewed extensively [5,6,12], but understanding of developmental arrest in oviparous reptiles that do not provide parental care after oviposition is limited. We present this information here and encourage future research to investigate the varying embryonic and early ontogenic strategies that are employed by different reptiles in response to their ecological demands.

(a) The environmental influence on embryonic development

Embryonic development occurs inside the amniotic egg (figure 2), which is composed of yolk and albumin encased in a porous, proteinaceous eggshell. The proteins produced in the mother's oviduct largely determine the egg environment, with yolk acting as the primary source of energy and albumin providing water and protein necessary for development [13,14]. The composition
and function of the egg components change throughout the developmental period, and as a whole, the developing egg (embryo and its extra-embryonic membranes) interacts with the external environment [13,15].

In the oviduct, the external environment consists of an oviducal material that bathes the egg and fills the shell pores. Proteins have been identified in the oviducal fluid of some reptilian species including the estuarine crocodile, *Crocodylus porosus* [16]; the American alligator, *Alligator mississippiensis* [17]; and the gopher tortoise, *Gopherus polyphemus* [18], although there is still little known of the composition and function of the majority of oviducal products. The transfer of growth factors secreted by the oviduct of gravid *A. mississippiensis* has been shown to affect embryonic development after being taken up by the egg [13,19]. However, the significance of the transfer of such materials is not fully understood.

After oviposition, development is affected by the surrounding nest environment and many studies have documented an environmentally induced variation in development caused by factors such as temperature [20,21]. Reptilian embryonic development and hatching phenotype are largely influenced by temperature and is known to determine sex in many species [22,23]. Temperature changes can affect the water potential of the egg and alter the metabolic rate and developmental period of a reptilian embryo [24]. This is evident through changes in embryonic oxygen consumption rates and the patterns of embryonic development [25,26].

### 2. SIGNIFICANCE OF ARRESTED DEVELOPMENT FOR OVIPAROUS REPTILES

Developmental arrest is a critically important reproductive strategy in the large range of egg-laying animals that provide no parental care after oviposition, because it provides their eggs a mechanism to respond to changing environmental conditions during embryonic development. From an ecological perspective, developmental arrest allows some embryonic resilience to adverse environmental conditions...
Pre-ovipositional arrest is generally observed during early embryogenesis but there are situations where it arises late in development. The tuatara, Sphenodon punctatus, and the common chameleon, Chamaeleo chamaeleon, are capable of arresting embryonic development during gastrulation, in addition to all marine and freshwater turtles that enter pre-ovipositional arrest as mid and late gastrulae, respectively, [11,28,40,62,63]. In contrast, squamate species including the North American iguanids, Urosaurus ornatus and Sceloporus undulatus, and the Indian agamid, Calotes versicolor, are capable of extended periods of embryonic development in the oviducts and embryos will arrest development if hatching is imminent and the eggs have not been laid [21,64].

(i) Causes of pre-ovipositional arrest

There are several morphological and physiological constraints likely to have given rise to pre-ovipositional arrest and the need to lay eggs at specific, mostly early, stages of development. In turtles, crocodilians and the tuatara, limited oxygen exchange might occur in the oviduct as a result of shell calcification and the shell pores filling with oviducal fluid [21]. Pre-ovipositional calcification of turtle eggs becomes complete when the embryos reach gastrulae and enter pre-ovipositional arrest approximately 7 days after ovulation [31]. Similarly, calcification of the eggshell is extended in squamate species that arrest during late developmental stages and completes just prior to arrest [65]. Eggshell calcification may infer a restriction on respiratory gas exchange needed for further growth of the embryo [32,33]. A reduction in eggshell thickness has been associated with extended egg retention and advanced embryonic development in reptiles, presumably because the availability of O₂ to the embryo is enhanced [31,66]. However, instances of eggshell thickening associated with extended egg retention in turtles have also been reported [67]. Additionally, the eggshell provides a source of calcium for developing turtle embryos and reducing the degree of eggshell calcification in order to achieve greater O₂ exchange may decrease embryo fitness [21,68].

The embryonic vitelline membrane and inner shell membranes of the egg must adhere to one another after calcification of turtle and crocodilian eggs to allow gas exchange.
diffusion and embryonic growth. Membrane fusion can only occur after oviposition because it is likely that the connection would rupture when eggs are jostled during oviposition, leading to embryonic mortality [21,69]. Vertical or horizontal movement of turtle eggs after membrane adhesion can cause movement-induced mortality [69–71], which has also been demonstrated in crocodilian embryos between 2 and 9 days after oviposition [22,61]. The presence of viscous oviducal secretions surrounding the egg and filling its pores inhibits formation of a respiratory surface that allows efficient gas diffusion [34,72].

Currently, there are no published data supporting the suggestion that hypoxia within the reptilian oviduct induces pre-ovipositional arrest [21,35,36]. Intra-oviducal oxygen tensions also remain unknown in reptiles, although there have been studies that estimated the oxygen tension in utero of the eastern fence lizard, Sceloporus undulatus, to be hypoxic [37,38]. Hypoxia is known to induce developmental arrest in budding yeast, Saccharomyces cerevisiae, and embryos of the nematode Caenorhabditis elegans, zebrafish, Danio rerio, and mouse, Mus musculus [73–76]. Hypoxia also maintains arrest in several species of short-lived fishes [77]. Oviducal oxygen tension has been measured at 37 mm Hg (5.3% O2) during the formation of pre-implantation blastocysts in pregnant hamsters [78]. These comparative findings suggest that a hypoxic environment within the oviduct of oviparous reptiles is likely and that a lack of oxygen may induce and/or maintain pre-ovipositional arrest.

Hypoxia causes the upregulation of the insulin-like growth factor binding protein (IGF-BP-1) [79–81] that binds to insulin-like growth factors (IGFs) and inhibit their activity. IGFs are greatly involved in embryonic metabolic activities and regulate all aspects of development and growth [82]. Altered expression of IGF proteins affects embryonic development in guinea pigs [83] and binding of IGFBP-1 to IGFs during hypoxia inhibits growth and causes developmental arrest in zebrafish embryos [84]. It is unknown whether this occurs in reptilian embryos that experience pre-ovipositional arrest, but it is plausible that oviducal hypoxia may cause expression of IGFBP-1 and thus hypoxia may influence developmental arrest. After oviposition, increased oxygen levels or natural degradation of IGFBP-1 may then allow IGF activity and resumption of development.
egg incubation when nesting occurs during temperate spring and summer in ephemeral suitable habitats [42]. On the other hand, developmental arrest in turtle species that nest in tropical and sub-tropical regions is most likely dictated by the seasonality of rainfall rather than temperature [4,11,42]. For example, pre-ovipositional arrest persists in embryos of the northern long-necked turtle, *Chelodina rugosa*, when eggs are laid underwater during the wet season. Embryos remain as gastrulae in response to underwater hypoxia until they are exposed to atmospheric oxygen when the nests dry out during the dry season [43]. A modified vitelline membrane in the eggs of this species prevents excessive water uptake during submersion [39]. The entire ontogeny of *C. rugosa* can be linked to environmental parameters with rainfall and hypoxia being involved in not just pre-ovipositional arrest in early embryos [99].

(ii) Post-ovipositional embryonic diapause

Embryos from several chelonian and chamaeleonid lizard lineages undergo a period of post-ovipositional embryonic diapause while they are still gastrulae [42,100]. Embryonic diapause is observed in the common chameleon, *C. chamaeleon*, the veiled chameleon, *C. calyptratus* and the Indian chameleon, *C. zeylanicus*, which lay eggs during temperate and subtropical autumn that take up to a year after oviposition to hatch because they arrest during cold winter months [40,44,101]. Embryos of the jewelled chameleon, *Furcifer lateralis* and the panther chameleon, *F. pardalis*, may also experience periods of embryonic diapause during the cooler dry season because they are laid during the wet season and do not hatch until the following wet season [45,46,47,102]. Embryonic diapause is an obligate part of the life cycle for each of these species, although resumption of development is facultative and does not appear to be linked to a mandatory environmental cue like those needed during embryonic diapause in some turtle species [29]. However, the length of time that embryonic diapause persists after oviposition has been linked to temperature in some chameleon species [29,40,101].

Australian broad-shelled turtle, *Chelodina expansa*, embryos also typically remain arrested after oviposition for several weeks before recommencing development briefly and entering a secondary state of embryonic diapause prior to somite and vitelline development, which persists throughout the winter period [29,42]. This type of embryonic diapause occurs after white spot development and is seen in healthy embryos that are in an environment which would normally promote active development [4]. Embryos in this state need to be exposed to an obligatory trigger to recommence development and in the absence of this cue, embryos remain arrested and eventually die [4].

Freshwater turtle embryos that show embryonic diapause take longer to reach specific development stages than those that do not when incubated at the same temperature [4]. The occurrence of embryonic diapause can be identified by comparing the developmental schedules of siblings or embryos of closely related species and recognizing whether pre-somite development is extended in relation to the remainder of development (i.e. whether development occurs in the expected timeframe) [4]. Five developmental stages are used to draw comparisons based on a determined developmental series [103].

These include the appearance of blood islands (stage 5), development of the haemodisc (vitelline circulation at stage 8), pigmentation of the eye (stage 12), pigmentation of the body (stage 20) and hatching (stage 26). The appearance of the white spot has also been used as a defining developmental stage [72,100]. These aforementioned stages can be observed in many species through non-destructive candling techniques and so make development in viable eggs relatively simple to assess [104].

In warm temperate regions, where oviposition generally occurs in autumn, embryos enter a state of embryonic diapause during the winter months and for some species such as the striped mud turtle, *Kinosternon baurii*, it has been described as seasonally dependent [4,42]. *Kinosternon baurii* females nest twice yearly during both spring and autumn, but embryos from the same females that are laid during both seasons only express embryonic diapause during autumn, suggesting that the occurrence of this reproductive characteristic is dependent on some external cue [42]. These embryos require a period of chilling to terminate embryonic diapause and resume development to hatching. Unchilled eggs of *D. reticularia*, the ornate wood turtle, *Rhinoclemmys pulcherrim*, and the Indian black turtle, *Melanochelys trijuga*, also require this stimulus, and they will remain arrested and subsequently die in its absence [11,25]. The same is true for *C. expansa* embryos, which require a chilling period during their developmental cycle in order for eggs to recommence development after embryonic diapause [29].

The ‘decision’ of whether or not an embryo expresses embryonic diapause is thought to occur during late oogenesis (between ovulation and fertilization) in *K. baurii* [42]. If females underwent chilling during this period, their embryos did not express embryonic diapause post-oviposition, but the embryos of unchilled females did [42]. This suggests that expression of embryonic diapause in embryos is determined by the temperature that mothers experience during late oogenesis, supporting the theory that maternal experience may influence the phenotype of their young [88,89].

The presence of embryonic diapause is characterized by annual wet–dry seasonality rather than by the temperature in sub-tropical and tropical locations [41]. These regions have seasonal rainfall patterns with minimal annual variation in temperature. In these areas, early incubation is spent in embryonic diapause during the wet season and the beginning of the dry season with hatching occurring the following wet season. In Pacific coastal regions of Mexico and Central America, turtle species including *Kinosternon*, *Staurotypus* and *Rhinoclemmys* express embryonic diapause in relation to seasonal rainfall, the same is also true for the north Australian snapping turtle, *Elseya dentata*, that lays its eggs during the austral wet season [42,105]. By laying eggs during the wet season, reptilian females may free themselves from the burden of carrying eggs for extended periods until suitable nesting environments are available. Additionally, it may also allow them to exploit the abundant food resources existing during these periods and thereby to produce subsequent clutches, assuming that successive clutches are not ovulated immediately after oviposition [43]. Likewise, hatching emergence during wet seasons may infer an advantage for the hatchlings because prey abundance is greater [48].
(iii) Cold torpor
Cold torpor is the facultative suspension of development that embryos use to survive brief periods during temperatures that are too low to support developmental requirements [4,11,50]. The embryos of most species of bird and reptile are capable of using this type of arrest at any stage during incubation, although others such as the montane lizard, *Acritoscincus duperreyi*, can only enter cold torpor when they are full-term embryos and not during earlier stages of development [49]. It is a direct response to unfavourable weather and in many cases, it protects embryos for varying durations at below-critical temperatures, although the chances of mortality increase with increased duration spent in torpor [4,51]. Avian species can typically only endure several days in torpor, whereas reptile embryos can withstand weeks [4]. Cold torpor can maintain pre-ovipositional arrest in turtle embryos [52] and in avian species that do not use pre-ovipositional arrest, torpor can prevent development of embryos after oviposition until all eggs in the clutch have been laid [8]. This allows the synchronization of development and hatching of the clutch [7].

(iv) Delayed hatching and embryonic aestivation
Delayed hatching and embryonic aestivation are strategies employed to prolong the residence of an embryo within the egg in response to adverse environmental conditions [4,42]. During both forms of arrest, the embryo is in the final stages of development and typically remains within an unpipped egg, although some species of bird may pip the egg before delaying hatching for up to a week [4,106]. Delayed hatching and aestivation differ only in the duration and degree of metabolic downregulation, so they are generally not distinguished in the literature. Delayed hatching occurs in avian, crocodilian, squamate and chelonian groups and although it may persist for several weeks in reptile eggs, it is usually brief in birds [4,55]. In contrast, aestivation is a late-embryonic dormancy that may last for weeks or months and empirical evidence of its occurrence has only been documented in turtles, although it may also occur in crocodiles and lizards [55,56,57,107]. Both delayed hatching and aestivation allow protection of young during unfavourable conditions and also permit synchronization of hatching when environmental surroundings promote optimum survival [4,53,57].

Generally, approximately 2 days before a turtle embryo pips the shell, the amnion ruptures inside the egg and the chorio-allantois moves posterior to the embryo, revealing the head and forelimbs and so freeing the embryo to pip the shell [11]. At the time of hatching, a yolk sac will also be external to the plastron [4,108]. However, when an embryo delays hatching, there is usually a greater delay in pipping after rupture of the amnion and movement of the chorio-allantois, in addition to internalization of the yolk reserve. Yolk absorption and final assimilation of any blood from the chorio-allantois signal the completion of embryonic development in turtles and crocodilians.

Turtle species including the pig-nosed turtle, *Carettochelys insculpta*, *K. scorpioides* and *K. flavescens* gradually enter aestivation after the chorio-allantois parts and migrates, such that it no longer covers the body of the turtle inside the eggshell [4]. Aestivation is sometimes considered an extension of delayed hatching if optimal conditions for hatching do not arise. The prolonged incubation period associated with aestivation generally involves the downregulation of metabolic processes and the subsequent decline in oxygen consumption rates to extremely low but steady levels [4]. Aestivation can extend for periods up to 116 days in *K. scorpioides* and 232 days in *K. flavescens* embryos if a stimulus to pip remains absent. In the former species, oxygen consumption can decrease to approximately 22 per cent of the peak rate [4]. Oxygen consumption rates of *C. insculpta* can decline to one-third of the peak rate when experiencing delayed hatching and aestivation at 30°C [109]. During these periods, the fully developed mature hatchlings of *C. insculpta* can remain within the egg for up to 59 days, after which the yolk reserve becomes depleted [57,109]. Extended aestivation periods potentially result in the weakening of the embryo and increase the likelihood of mortality if all energetic reserves have been exhausted and a stimulus to pip is still absent [4,41]. Not all species of turtle are capable of aestivation and when late-term embryos of the painted turtle, *Chrysemys picta* and *C. serpentina* are exposed to conditions that induce aestivation in other aestivating species, development and hatching are hastened because of the unfavourable conditions [4].

The proximate mechanisms associated with the metabolic changes observed during delayed hatching and aestivation, which appear to be actively regulated independent of temperature, are poorly understood. However, a possible factor involved in the process may be thyroid hormone [54,110]. Thyroid hormone is vital for the growth and function of most vertebrate tissues and is capable of acting on both metabolic and non-metabolic pathways to influence embryonic tissue accretion and differentiation [111]. Thyroid hormone is also involved in energy metabolism and has been linked to the hatching process in chickens [112,113]. Increased thyroid hormone levels are known to stimulate hatching, and production of this hormone is induced in hypoxic conditions [112]. Hypoxia is the proximal cue known to trigger aestivating *C. insculpta* eggs to rapidly hatch, although whether this process involves thyroid hormone remains to be tested [57].

Environmentally induced cues have been explained to terminate delayed hatching and aestivation. Both processes were originally thought to occur in response to hot environments, although recent research suggests that delayed hatching and aestivation are more likely strategies to coincide hatching with wet season productivity [41,57]. In doing so, delayed hatching and aestivation allow hatchlings to exploit early wet-season productivity to promote growth and survival rather than to avoid any embryonic stress that might be caused by late dry-season conditions [4,106,109]. As mentioned previously, hypoxia induced by complete water inundation of *C. insculpta* eggs is the trigger that induces explosive hatching during periods of heavy rainfall [57,109]. Eggs of hole-nesting crocodilians may also undergo this process based on similar nesting and hatching patterns, but more research is needed to confirm this [107]. Additionally, heavy rainfall at the onset of the wet season may trigger hatching in the Fiji-crested iguana, *Brachylophus vitilis*, but it has not yet been determined whether extended incubation periods are associated with delayed
hatching and aestivation, or embryonic diapause in this species [55,114]. Evidence is also emerging which suggests that there are additional mechanisms, other than fluctuations in nest gas concentration, that trigger eggs to hatch synchronously after undergoing a period of quiescence. Vibration-induced hatching, triggered by the vibrations of some eggs hatching earlier than others, has been proposed [55]. In addition, audible triggers such as the pipping of eggs or vocalizations by embryos is plausible considering that sibling communication has been identified in eggs of both crocodilian and some avian species [54,58]. However, all of these hypotheses remain to be fully tested.

4. CONCLUSIONS AND DIRECTIONS FOR FUTURE ENQUIRY

All forms of arrested development synchronize the developmental timing of turtles in accordance with conditions that increase the chances of embryonic survival in the absence of parental care [115]. Developmental arrest allows turtles to nest for longer periods throughout the year and also permits hatching turtles to emerge when food may be more abundant. Early stage pre-ovipositional arrest guarantees that embryos avoid movement-induced damage during oviposition and ensures that eggs are laid prior to the resumption of development. Pre-ovipositional arrest is universal in turtles and occurs in many other species of reptile, but it is the least studied form of arrest. The majority of studies investigating strategies to arrest embryonic development have focused on the time between oviposition and hatching, thus overlooking the period of development inside the mother. The evidence presented in this review suggests that the conditions experienced by the embryo while inside the oviduct influences the ability of the embryo to respond to environmental factors including oxygen tension, temperature and moisture. The key to fully understanding developmental processes includes addressing maternal influences and priority should be placed on understanding how the relationship between maternal factors and environmental factors affect embryonic development.

Embryonic diapause, cold torpor, and delayed hatching and aestivation allow avoidance of unfavourable environmental conditions that would otherwise have a negative effect on successful growth and development of the embryo. The majority of work on these topics has been undertaken in an artificial environment in the laboratory and more field-based research is needed to fully understand how processes such as developmental arrest are influenced by natural environments. In addition, the developmental chronology of many oviparous animals remains unknown and by identifying how embryos develop, comparisons and conclusions can accurately be drawn between groups that employ different developmental strategies to deal with a changing environment. Furthermore, future investigations should identify whether embryos will preferentially arrest at certain developmental stages more than others. Although the assumption that developmental arrest occurs in response to environmental factors associated with an embryo's external environment is widely held, the cues that cause the onset or cessation of arrest still remain unclear in most cases. Future studies in the field of reptilian embryology need to identify how environmental ecology can dictate the evolution of developmental processes associated with embryonic development and ontogeny [116,117]. In doing so, we will better understand how the natural environment has shaped the evolutionary history of a wide range of species and help uncover some of the secrets associated with terrestrial invasion and evolution of the incredible ecological adaptations that have come about in response to challenges associated with laying shelled eggs on land [118].

Overall, the topic of embryonic developmental arrest warrants much more attention than it has previously received. Understanding the processes involved in arrest has implications for conservation biology and population recovery of critically endangered egg-laying animals because it is essential to understand the natural history of a species before attempts can be made to artificially incubate eggs successfully. In many captive situations, it is unlikely that eggs will survive to hatching if the triggers for breaking periods of arrest are unknown. In addition, understanding what induces a state of early stage embryonic arrest may allow the successful transport of eggs without risk of movement-induced mortality, thus allowing for *ex situ* research on a multitude of different species. Finally, it also offers a fundamental understanding of the diversity of reptilian developmental processes and their consequences.

REFERENCES


49 Radder, R. & Shine, R. 2006 Thermally induced torpor in fullterm lizard embryos synchronizes hatching with


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**Proe. R. Soc. B** (2012)


