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Patterns of embryonic development

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The embryology of reptiles is an important component of two emerging research areas. One area is the result of the marriage of molecular and developmental biology. Molecular biology has provided the tools to demonstrate the genetic commonality of developmental patterns, and thus provides novel insights into the origin and maintenance of structural diversity across the animal kingdom (Raff, 1996). Hox genes are the master regulatory genes whose products are expressed in specific locations on the long axis of embryos and so are the cornerstones of this commonality. Hox genes, and the cascade of other regulatory and structural genes that they affect, specify the axial pattern (trunk regionalisation and limb position) during embryonic development. Mutations to Hox genes, thus, can alter the number and position of structures and even determine whether some particular structure develops at all. For example, studies on limb development have demonstrated that modifications to Hox and associated regulatory genes provide a mechanistic explanation for the absence of forelimbs and the extreme reduction in size of the hindlimbs of pythons, and squamates with other reduced limbs (Cohn and Tickle, 1999).

The second area is the evolutionary ecology of development. This area emerged with the recognition that environmental conditions during incubation affect phenotypes of post-hatching reptiles and had its conceptual origin in the observation that the sex of some reptiles is determined by incubation temperature (Janzen and Paukstis, 1991b; Chapter 9). Subsequent research has documented that numerous other phenotypic traits of post-hatching reptiles are affected by the embryonic environment (see Chapter 10). The critical issue at this time, and one that proves to be extremely difficult to address, is the potential adaptive value of such phenotypic variation (see Chapter 8).

Classic embryology provides the conceptual foundation of both research areas. This review focuses on reptilian embryology with regards to its relationship with and potential contributions to molecular, physiological, ecological, and evolutionary studies. The major premises are that a very broad overview of reptilian

development would be useful for experimentalists in general and that the embryo *per se* is an under utilised resource for ecological and evolutionary studies. For example, investigations into the effects of incubation conditions on post-hatching phenotypes typically consider the egg as the experimental unit and that oviposition and hatching are the respective beginning and end points of development. This 'white box' approach involves experimental manipulation without regard to the developing embryo inside the egg. While this approach has been productive, it has two general kinds of problems. One problem is that it may limit the insights that can be gained from an experiment. For example, because very small gradients in temperature across the shell membrane can dramatically affect the water relations of eggs (Ackerman, 1994; Chapter 2), pairing data on water uptake and embryo mass/metabolism would provide a more precise understanding of the dynamics of water balance during incubation. A more serious problem is that the lack of information about the stage of the embryo could result in experimental bias. For instance, oviposition by squamates occurs at a wide range of developmental stages among species (Andrews and Mathies, 2000), and variation among clutches of the same species can also be substantial (Braña *et al*., 1991). In the absence of information about the stage of the embryo, treatments could be imposed at various times relative to the temperature-sensitive period for a particular trait. The resulting variation could reduce the ability to detect treatment differences for intra-specific comparisons or imply differences among species that don't exist.

The main objectives of this chapter are to: 1) review both qualitative and quantitative aspects of the embryonic development of reptiles; and 2) review developmental arrest, emergence from the egg and the nest, and factors affecting hatchling size, i.e. developmental phenomena that do not involve differentiation and growth of the embryo *per se*. Special attention is paid to information published since Deeming and Ferguson's (1991e) review of these subjects. Information will be provided that broadly facilitates comparative studies of embryonic development and encourages more experimentalists to open the 'white box' and to discover what embryos are actually doing during their experiments.

Throughout the chapter, the term reptile will be used in its common usage sense, i.e. turtles, crocodilians, tuataras, snakes, and lizards. A caveat concerns the treatment of birds, which are ultimately part of the reptilian radiation. The large size of the group and the unique derived features of birds related to endothermy and flight, however, have meant that birds have their own specialised literature with regards to development. Moreover, the development of birds is covered in a recent review (Deeming, 2002a). Birds will thus be included in this review only when a critical comparative purpose is served.

Embryonic development: commonality among reptiles

Development of the reptilian embryo *per se* involves two distinct but related processes. One is differentiation, i.e. the origin of tissues and organ systems. The other is growth, i.e. the increase in the size of the embryo. Integral to both differentiation and growth of the embryo is the parallel development of the extra-embryonic membranes. While these three processes are highly integrated, they are often presented as stand-alone components of development. Therefore, development is described here in a general way in an attempt to illustrate the temporal relationships of these major developmental processes.

General descriptions of reptilian development will be based on the development of the domestic fowl (*Gallus gallus*) because parallel sets of data are available for the differentiation and growth of the embryo and for the development of extra-embryonic membranes (Brody, 1945; Lillie, 1952). By contrast, such a comprehensive data set is not available for any reptile species with the possible exception of the crocodilians *Alligator mississippiensis* (Ferguson, 1985; Deeming and Ferguson, 1989a) and *Crocodylus johnstoni* (Manolis *et al*., 1987; Webb *et al*., 1987c). Development of reptiles and birds is highly conservative (Stewart, 1997; Ricklefs and Starck, 1998). This means that the general patterns of development discussed are representative. This conclusion is supported by the quantitative comparisons in the following section of this review. Development is typically documented in 'stages' that describe the characteristics of embryos on the basis of their morphology but not necessarily related to any particular time during incubation. As will be demonstrated the exact shape of the relationships between stage and time and mass and time are affected by incubation temperature (see below). Table 4.1 provides information about the sequence of selected developmental events and Figure 4.1 provides a visual summary. General sources of descriptions of embryonic development include Lillie (1952), Romanoff (1960), Gans *et al*. (1985), Gans and Billett (1985), Mossman (1987), and Stewart (1993, 1997). The intent of the following brief summary of reptilian development is to provide an overview of common features of development of reptiles and birds and to provide the basis for the quantitative analyses that follow.

The description of differentiation starts with neurulation as a common point of reference because it is more often assessed in embryological studies than earlier stages. Moreover, events during neurulation are relatively distinctive and would thus be of utility to non-embryologists attempting to stage embryos for evolutionary or ecological studies. During neurulation, the tissues of the embryo *per se* are separated from the tissues that form three of the four extra-embryonic membranes by the formation of the head and body folds.

Table 4.1. Stages (first appearance) used to quantify the temporal pattern of development, major developmental events (following El Mouden *et al.*, 2000), and representative organ systems in place during these events. Dufaure and Hubert (1961) stage numbers (DH) are used for consistency.

Diagnostic stages (DH number)	*Event (DH stages)*	*Events:*
Neural groove (10) Somites (16)	Neurulation (10-20)	Germ layers, brain differentiation, amniontic cap, heart and blood vessels
Torsion initiated, head on left side (21) Allantois bud (25) Torsion complete, embryo's on its left side (25) Limb buds (27)	Organogenesis (21 – 29)	Amnion, chorion and yolk sac completed, all major organ systems in place (circulatory, nervous, digestive, respiratory, body etc.)
Apical epidermal ridge forms on limb bud (30) Digits visible in limb paddle (34)	Early growth (30-34)	Chorio-allantoic membrane completed, limbs and feet, gonads, hemi-penes
Lower jaw reaches end of snout (35)	Late growth (35-40)	Increase in mass, scalation, pigmentation, eyelid

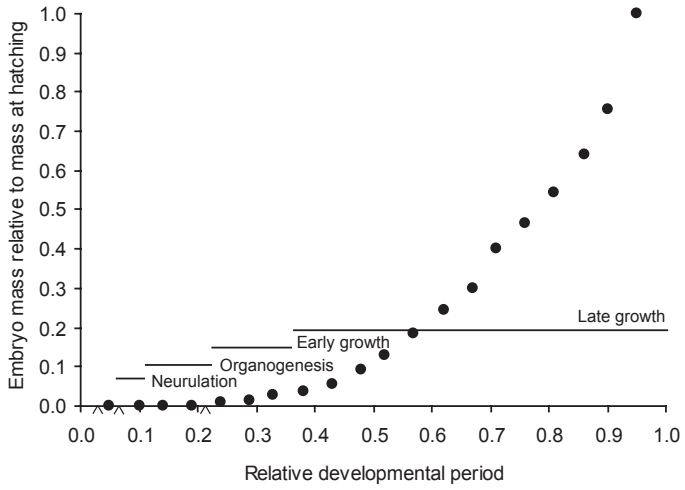

Figure 4.1. Differentiation and growth (live mass) of the chick embryo. The axes are the proportion of final hatchling mass obtained (Y) and the proportion of total development period completed (from ovulation). The black dots represent consecutive days of development for the fowl embryo. Gastrulation occurs from day 0 to day 1. The shape of the growth curve and associated stages of development are typical of reptiles and birds. The developmental events associated with neurulation, organogenesis, early growth, and late growth are outlined in Table 4.1. The three carets (^) on the X axis, from left to right, indicate the mean stage at oviposition of turtles, crocodilians, and squamates respectively.

The first step in the formation of the extra-embryonic membranes is the expansion of tissue layers (ectoderm, endoderm, and mesoderm) peripheral to the embryo over the surface of the yolk. When the mesodermal layer splits to form the extra-embryonic coelom, the inner vascularised mesoderm-endoderm layer (splanchnopleure) becomes the definitive yolk sac. The outer ectodermal-non-vascularised mesodermal layer (somatopleure) in conjunction with ectoderm forms the amnion and the chorion. The head and body folds (somatopleure) that surround the embryo form the amnion. Somatopleure tissue that extends beyond the sero-amniotic connection (i.e. the junction of the two folds of the amnionover the embryo) lines the inner surface of the shell, forming the chorion. Formation of the yolk sac, chorion, and amnion occurs concurrently with the latter stages of neurulation and early stages of organogenesis. The amnion completely surrounds the embryo about the time that torsion is complete and the embryo is lying fully on its left side; the chorion and yolk sac reach their final coverage of the inner shell and the yolk sac, respectively, somewhat later in development. The yolk sac is vascularised and so it may serve to provide oxygen to the embryo early in development. At the time when the yolk sac is the only vascularised membrane in contact with the shell, however, embryonic demands for oxygen are very low. The fowl embryo, for example, is only about 0.1% of the live mass of the term neonate at this time (Brody, 1945).

The allantois is the last extra-embryonic membrane to form. It emerges as an outgrowth of the hindgut (endoderm and mesoderm) concurrent with or shortly after completion of the amnion and somewhat prior to or concurrent with the formation of limb buds. It expands into the extra-embryonic coelom where it contacts and fuses with the chorion, forming the vascularised chorio-allantoic membrane (CAM) that lines the inner surface of the shell. The CAM provides a large surface for gas exchange through the overlying shell and supplants the yolk sac as a respiratory surface (although the yolk sac may not have a significant respiratory function in some species). The temporal pattern of CAM development is not well known and no general pattern evident in what little data is available. For example, in the fowl (with an incubation temperature of 38°C), it covers most of the inner shell about 40% through incubation when the embryo is about 5% of the mass of the term embryo. In *C. johnstoni* eggs incubated at 30°C, the CAM is complete at about 70% of the incubation period, when the embryo is 25% of its mass at term (Manolis *et al*., 1987). In *Sceloporus* lizard eggs incubated at a mean of 27°C, the CAM covers 30–50% of the inner shell at oviposition when embryos are about 10% of the mass of the hatchling (Andrews, 1997) and when 25–30% of the developmental period has been completed (DeMarco, 1992, 1993). The relative area of the CAM is greater for *Sceloporus* eggs that have high water uptake than in eggs with low water uptake, despite comparable embryo stages. This observation

suggests that water uptake and expansion of the allantois may drive the growth of the CAM rather than the immediate oxygen needs of the embryo (Andrews, 1997).

Embryonic differentiation and formation of the extra-embryonic membranes are largely complete quite early in the developmental period and well before any appreciable growth of the embryo has occurred (Figure 4.1). In general, differentiation of tissues, organ systems, including the gonads and body structures, takes place in the first 30–40% of the developmental period. At this time, embryos are recognisable as birds, lizards, turtles, *etc*., even though they have reached less than 5% of their mass at hatching.

Once the embryo and its supporting structures are in place, growth becomes the most conspicuous feature of development (Figure 4.1). Dramatic increases in the size of the embryo are accompanied by changes in the size and configuration of the extra-embryonic membranes as judged by the volume of the fluid compartments of the egg (Deeming and Ferguson, 1991a; Deeming, 2002b). The surface area and volume of the yolk sac first increase somewhat to accommodate the influx of water from the albumen (in turtles, crocodilians, and birds) or from the environment (in squamates) and then steadily decrease as nutrients are transferred from the yolk to the embryo. The area of the amnion increases in size to accommodate the growing embryo; the volume of the amniotic fluid increases and then declines towards the end of incubation. The volume of allantoic fluid exhibits the same pattern as the amniotic fluid with an initial increase and a latter decline, presumably as water is transferred to the embryo. The area of the inner surface of the egg limits the area of CAM. For reptiles with rigid-shelled eggs, the maximum CAM area is fixed at oviposition by the size of the egg. By contrast, for reptiles with flexible-shelled eggs, the maximum CAM area depends on the amount of water uptake and the concomitant increase in the surface area of the egg. For example, an increase in egg mass of 2–5 fold (typical of squamate eggs) would be associated with an increase in shell area of about 1.5–3 fold. Unfortunately complete descriptions of the extra-embryonic membranes and fluids for reptiles are only available for crocodilians (Manolis *et al*., 1987; Deeming and Ferguson, 1989a). There is a need for similar data to be collected in other reptile groups.

Embryonic development: diversity among reptiles

A recent phylogenetic treatment of basal Reptilia identified two major sister groups: turtles plus archosaurs (sister-groups to one another) and *Sphenodon* plus squamates (Rest *et al.*, 2003). This phylogenetic dichotomy is correlated with shell structure and with the major sources of calcium and water for the developing embryo. Archosaurs (crocodilians and birds) and some turtles are characterised by rigid heavily mineralised shells while lepidosaurs (sphenodotids and squamates) and

some turtles are characterised by pliable-shelled eggs that have a thin mineral layer (Packard and DeMarco, 1991). Members of two sub-families of the lizard family Gekkonidae are an exception within lepidosaurs because their rigid, heavily mineralised shells are derived within the Squamata. Variation in shell type also occurs within turtles but a phylogenetic signal is not obvious because most lineages exhibit both shell types (Iverson and Ewert, 1991). The putative sister relationship of turtles with archosaurs, however, would suggest that rigid-shelled eggs are ancestral.

The phylogenetic dichotomy within the reptiles is associated with a dichotomous pattern of provisioning of two fundamental nutrients within the egg. One such nutrient is calcium (Packard, 1994; Packard and Clark, 1996). For turtles, crocodilians, and birds, the major source of calcium for embryonic development is the thick mineral layer of the shell. During the growth phase of development, calcium is mobilised from the eggshell to supplement the modest amounts of calcium originally deposited in the yolk. At hatching, the residual yolk of turtles contains very small amounts of calcium. By contrast, the residual yolk of archosaurs contains large quantities of calcium that presumably can be used by the neonate after hatching. For squamates, the major source of calcium for embryonic development is the yolk. The shell in some lizards, however, can contribute a substantial amount of calcium, for example in the lizard *Iguana iguana*, 47% of the calcium incorporated into the embryo is derived from the shell. While this pattern is related functionally to shell type, phylogeny appears to override shell type, at least in the case of turtles, where the shell provides the majority of calcium used by the embryo even for species with pliable-shelled eggs.

The other nutrient that is provisioned along phylogenetic lines is water. For turtles, crocodilians and birds, eggs are provisioned with a thick layer of albumen that serves as the source of water for the developing embryo (Table 1.1). For birds, whose nests are generally exposed to the air, eggs are provisioned with sufficient water to compensate for water loss through evaporation. For crocodilians and turtles, whose eggs are in subterranean nests where relative humidity is high, eggs may either take up or loose water depending on the physical conditions of the nest. For reptile species with rigid-shelled eggs, water exchange is negligible. For turtles with pliable-shells, water exchange can be appreciable. Snapping turtle (*Chelydra serpentina*) eggs, for example, may lose or gain as much as 30% of their original mass (Packard, 1999). Shell type, however, is not associated with the amount of albumen in turtle eggs at the time of oviposition (Stewart, 1997; Table 1.1). By contrast, squamate eggs are initially provided with little or no albumen (Chapter 3), and water uptake from the environment is necessary for successful development (Chapter 1). The mass of lepidosaur eggs increases 2–5

fold during incubation. Some adaptive variability apparently exists with regards to initial water stores in quamate eggs. For example, eggs of the Galapagos land iguana (*Conolophus subcristatus*) have unusually large amounts of albumen, a putative adaptation to extremely dry incubation environments where a typical squamate egg would not be able to take up enough water from the environment to survive (Tracy and Snell, 1985). However, it may also reflect the relatively large egg mass in this species. Data on the effects of size on egg composition in different squamate species would be an interesting area of research.

The stage of development at oviposition also differs among reptilian groups. Turtles, crocodilians, birds, and sphenodontids all lay their eggs when embryos are at very early stages of development: gastrulae, neurulae, blastulae, and gastrulae, respectively. During abnormally extended egg retention in turtles, development of the embryo remains arrested at the gastrula stage (see latter section on developmental arrest). The embryo continues to develop during abnormally extended egg retention in crocodilians and birds but development is abnormal (Ferguson, 1985). By contrast, for the great majority of squamates, development proceeds normally while eggs are *in utero*, and by the time of oviposition, embryos typically have reached limb-bud stages (Andrews and Mathies, 2000). The range, however, is considerable. At the one extreme, shelled eggs of a handful of squamate species hatch after being retained *in utero* to near term whilst at the other extreme, eggs of some chameleons (e.g. *Chamaeleo calyptratus* and *Furcifer lateralis*) are laid when embryos are gastrulae (Blanc, 1974; Andrews and Mathies, 2000; Andrews and Donoghue, 2004). The availability of oxygen *in utero* may explain the amount of development that occurs prior to oviposition in reptilian eggs (Andrews and Mathies, 2000). Turtles and crocodilians have thick eggshells that would be especially resistant to gas exchange in the oviduct where pores in the shell are filled with fluid. On the other hand, most squamates have thin shells that are relatively permeable (Deeming and Thompson, 1991; Chapter 1). The ability to retain eggs that continue to develop may be the explanation, or part of the explanation, why viviparity has evolved so many times in the squamates, but not in turtles, crocodilians, or birds.

Comparative embryology

Differentiation is clearly continuous; for descriptive purposes, however, differentiation is broken into a series of stages, each characterised by the appearance of a defined morphological feature. The chronological sequence of stages for a particular species is its normal table of development. As such, because of the nature of development, stages are initially closely spaced in time because new features appear in close succession; as development proceeds, differentiation of new features gives way to increase in size as the dominant process of development

(Figure 4.1). Inspection of normal tables across the spectrum of reptiles shows that the general pattern of differentiation among reptilian groups is very similar, at least in qualitative terms, and especially so during early development (see reviews in Gans and Billett, 1985 and Gans *et al*., 1985).

The question addressed in this section is whether reptilian development is quantitatively similar as well. Two issues are of particular importance. The first concerns the effect of temperature during development. A number of authors have suggested that early stages are more sensitive to temperature than later stages (e.g. Yntema, 1968; Birchard and Reiber, 1995; Shine and Elphick, 2001), but the generality of this observation is unknown. The second issue concerns whether morphological features (stages) appear at the same sequence and at the same relative time during development. In a comparison of the development of a lizard with that of a sea turtle or a bird, would the embryos show the same or different developmental trajectories?

To make such quantitative comparisons, however, some specific requirements must be meet by the data. The most obvious is that normal tables of development must be available for species across the range of reptilian taxa. Critical, but less obvious requirements concern the information provided by the tables themselves. The same morphological features are not always used for staging. This means that stages available for comparative purposes are limited to a subset of the possible stages. Moreover, some tables start early in development and others relatively late in development; starting points are often associated with oviposition, and the amount of development completed before oviposition varies considerably among reptiles (Andrews and Mathies, 2000). Tables must thus be standardised to enable comparative analysis. Finally, a normal table that is well characterised in terms of morphological description is not in itself sufficient for comparative purposes unless it includes the temperature that embryos experienced during incubation and the time elapsed between stages. While comparisons made at the same standard temperature would be ideal, such data is not available and it is not likely such data could be collected. Mean incubation temperature ranges from less than 20°C for the tuatara (Thompson *et al*., 1996), to 35°C or more for other reptiles (Packard and Packard, 1988a). Finally, observations made at temperatures outside of the range in which hatching normally occurs are not appropriate for comparative purposes (e.g. Yntema, 1968).

Using the above criteria twenty normal tables were suitable for comparative analyses. They represent 14 species of lizard in five families, two species of turtle, three species of crocodilian, and the domestic fowl (Table 4.2). The staging table and stages numbers of Dufaure and Hubert (1961) for *Lacerta vivipara* were used

to standardise staging across species (hereafter abbreviated to DH). This table was used as a standard for two reasons. Firstly, stages cover the whole developmental sequence starting at stage 1 with the initiation of cell division and ending at stage 40 when hatching or birth occurs. Secondly, the Dufaure and Hubert (1961) table is the one most commonly used for staging lizard embryos, the group for which most data are available. Not all tables presented the same developmental features so stages were assigned to a sub-set of features that were common to the majority of the tables. For species with tables that included very early development, time = 0 was the beginning of neurulation (DH stage 10); subsequent developmental stages were recorded as the time in days from this stage. For species with tables that started later in development, time = 0 was the formation of the apical epidermal ridge on the limb bud (DH stage 30). These standardised times were used to determine the length of major developmental events (neurulation, organogenesis, early growth, and late growth; Table 4.1). Standardised times from neurulation to subsequent stages were divided by the time between neurulation and hatching/ birth to represent the relative time during development that various stages occurred. Body size influences many biological processes (Calder, 1984) and so embryo or species size *per se* could affect the pattern of development. Therefore, the mean live mass of hatchlings was used as an index of embryo size in statistical analyses. Hatchling mass was used because the size of the embryo changes drastically during development and because hatchling size is strongly correlated with adult size (Andrews, 1982). When the original paper did not present this information, other literature sources were used to obtain or estimate hatchling mass. The range of hatchling mass is so wide (0.2 to 92 g; Table 4.2) that estimation of hatchling mass for some species is unlikely to bias analyses.

Effect of temperature during development

For three of the four developmental intervals, the length the interval decreased with temperature (Figure 4.2, Table 4.3). Earlier stages were more sensitive to temperature than later stages (Chapter 5) therefore confirming reports of Yntema (1968), Birchard and Reiber (1995) and Shine and Elphick (2001). Temperature accounted for a significant amount of variation in the length of neurulation, organogenesis, and early growth, during roughly the first one-half of development (starting with neurulation). By contrast, the length of the late growth interval, or the latter half of development, was independent of temperature. This was true for both the entire data set, and for the sub-set of 10 species that provided the data for lengths of neurulation and organogenesis. This latter analysis was run to determine if the lack of temperature sensitivity in the entire data set was the result of species composition; the data set was dominated by lizards, while the subset of 10 species was more evenly balanced among groups. The rate of growth in mass also appears to be more sensitive to temperature early than late in development. For example,

up to an embryo mass of about one half of hatchling mass, growth of *C. johnstoni* embryos is a function of temperature, but during the latter half of development, growth is not (Whitehead *et al*., 1990). Similarly, growth of *A. mississippiensis* embryos is also more sensitive to temperature early than late in development (Deeming and Ferguson, 1989a).

Table 4.2. Data from normal tables for 20 species of reptile and one bird. Data are the durations of Neurulation (N), Organogenesis (O), Early Growth (EG), and Late Growth (LG) as defined in Table 4.1. Mass is mean hatchling mass and Temperature is mean incubation temperature.

Species	*Mass (g)*	*Temperature (°C)*	*Interval (days)*				*Source*
			N	*O*	*EG*	*LG*	
Turtles							
Chrysemys picta	5.0	22	10	13	47	22	15
Chelonia mydas	25.0	29	6	7	17	28	16
Lizards							
Chamaeleo calyptratus	0.6	28	8	16	15	71	1
Chamaeleo lateralis	0.2	21.5	35	28	35	67	2
Pogona vitticeps	3.0	28			22	43	1
Agama impalearis	2.0	30			12	37	3
Phyllodactylus marmoratus	0.5	25			26	48	4
Lacerta vivipara	0.2	27	7.4	9	7	16	5
Podarcis muralis	0.3	26			14	29	6
Urosaurus ornatus	0.25	27			17	24	7
Sceloporus scalaris	0.3	30			11	23	8
Sceloporus woodi	0.4	30			16	30	8
Sceloporus virgatus	0.4	25			19	42	9
Sceloporus undulatus	0.5	28			14	35	1, 10
Liolaemus tenuis	0.4	22			27	28	11
Liolaemus gravenhorsti	0.4	~25		14	9	23	12
Crocodilians							
Alligator mississippiensis	73.0	30	5	5	20.5	37.5	13
Crocodylus porosus	92.0	30	5	5.5	24.5	60	14
Crocodylus johnstoni	44.0	30	5	6.5	23.5	67	14
Birds							
Gallus gallus	40	38	1.3	2.4	3	13.5	17

Source: 1: Andrews (unpublished observations); 2: Blanc (1974); 3: El Mouden *et al.* (2000); 4: Thompson and Russell (1999a); 5: Hubert (1985); 6: Dhouailly and Saxod (1974); 7: Mathies and Andrews (1999); 8: DeMarco (1993); 9: Andrews and Rose (1994); 10: Andrews (1999); 11: Lemus *et al*. (1981); 12: Leyton *et al*. (1980), Lemus (1967); 13: Ferguson (1985); 14: Webb *et al*. (1987c); 15: Mamoud *et al*. (1973); 16: Miller (1985); 17: Lillie (1952).

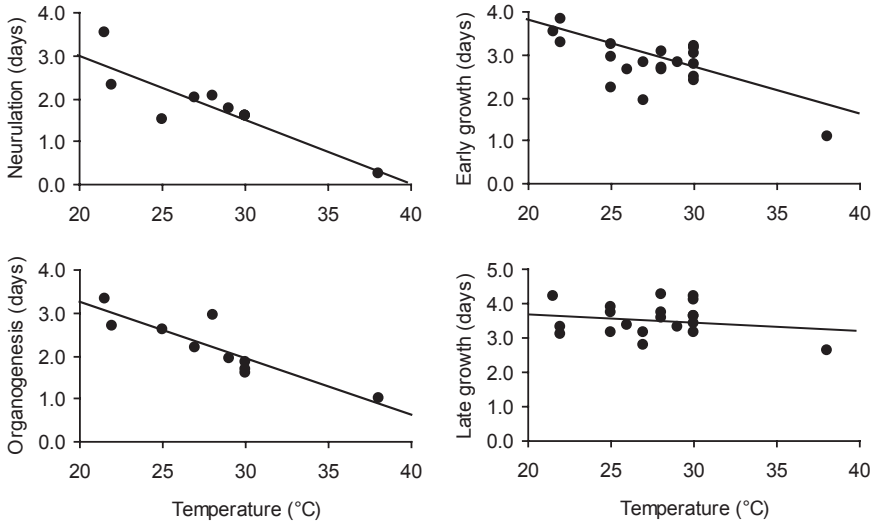

Figure 4.2. The relationships between the lengths of four major developmental intervals (natural log transformed) and incubation temperature for the species in Table 4.2. Regression statistics are presented in Table 4.3.

Table 4.3. Statistical comparisons of the length of four different intervals of development as a function of incubation temperature and hatchling mass using stepwise multiple regression models. Dependent variables were natural log transformed to linearise the relationship between the length of the interval (I) and temperature (T). Hatchling mass (H) did not make a significant contribution to the Neurulation or the Organogenesis models (p's = 0.70 and 0.08, respectively). The Early Growth and Late Growth models were run with all 20 species and with the subset of 10 species for which data were only available for Neurulation and Organogenesis. Temperature was the only significant variable for Neurulation and Organogenesis and entered first for the Early Growth Models where hatchling mass was also significant.

Interval (Y)	*Model*	*n*	*F*	*p*	r^2
Neurulation	Ln I = 5.795 – 0.143T	10	24.1	0.001	0.75
Organogenesis	Ln I = 5.798 – 0.130T	10	46.2	0.001	0.85
Early Growth	Ln I = 6.663 – 0.146T + 0.011H	20	16.1	0.001	0.49 (T), 0.65 (T+H)
	Ln I = 7.279 – 0.177T + 0.016H	10	11.5	0.006	0.45 (T), 0.77 (T+H)
Late Growth	None were significant	20	1.2	0.28	0.06
		10	0.7	0.43	0.08

The observation that temperature has the greatest effect on differentiation and growth of the embryo early in the developmental period means that the stage when experimental regimes are imposed (typically at oviposition) is thus critical for experimental purposes. For turtles and crocodilians, researchers are justified assuming common starting point for embryos as oviposition occurs when embryos are gastrulae and neurulae, respectively. By contrast, because most squamates oviposit relatively late during development, experimental studies on squamates could profit from an assessment of embryo stage at the time when observations are initiated or when individuals are shifted from one experimental thermal regime to another. Development is synchronised within clutches (Mathies and Andrews, 1995), so a sample of one embryo per clutch would provide this critical information while leaving the remainder of the clutch for experimental purposes.

The general pattern of differentiation is the same for eight species (only one crocodilian is illustrated) whose normal tables include early developmental stages (Figure 4.3). Differentiation, as judged by the progression of stages, is rapid initially but slows appreciably towards hatchling. Moreover, the rate of differentiation is temperature dependent. At low temperatures (e.g. *Chamaeleo lateralis*), early stages are well spaced in time and differentiation and early growth stages (prior to DH stage 35) are completed at about 60% of the development period. By contrast, at high temperatures (e.g. *G. gallus*), early stages progress rapidly and differentiation and early growth are completed at about 40% of the developmental period. Compression of differentiation with increasing temperature is illustrated by the negative regression between the proportion of the developmental period elapsed when the lower jaw is complete (PJAW, DH stage 35) and incubation temperature (T). This relationship is:

$$PJAW = 1.11 - 0.022\ T\ (F_{1,8} = 13.0,\ P = 0.007,\ r^2 = 0.62) \qquad [4.1].$$

Visual inspection of the data plotted in Figure 4.2 suggests that the bird embryo (at 38°C) simply represents the high temperature end of reptilian embryonic differentiation rates. This interpretation should be considered tentative, however, pending a statistical analysis that would contrast a range of reptilian and avian species at the same developmental intervals. Given that temperature sensitivity is stage dependent in reptiles, the rate of differentiation of reptiles and birds may be similar during early development (when corrected for incubation temperature), but diverge later in development. Such divergence would be in accord with the observation that birds have higher post-hatching growth rates than reptiles (Case, 1978).

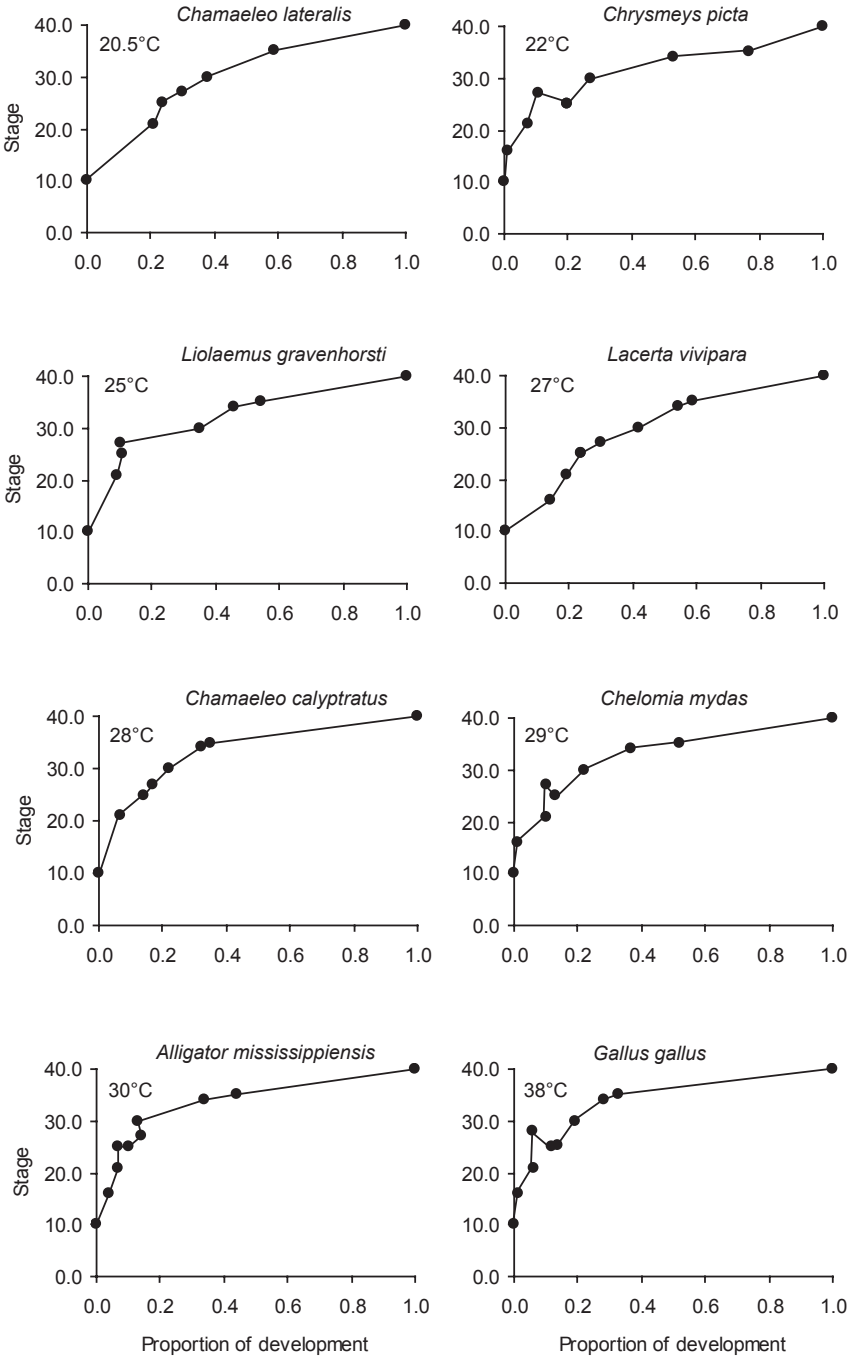

Figure 4.3. The relationship between stage and the proportion of development completed (from neurulation) for eight seven species of reptile and one bird. The graphs are presented in a sequence of increasing incubation temperature.

Effect of hatchling size on development

Embryo size, as indexed by hatchling mass, was not related to the length of neurulation and organogenesis (Table 4.3). Moreover, embryo size explained considerably less variation than temperature in the length of organogenesis. These results should not be interpreted as demonstrating that hatchling size has little effect on development. Given the strength of the temperature effect, the small number of species included, and the wide taxonomic range of species represented, the analysis is simply not sensitive to this variable. Similarly, from visual inspection of the data, the rate and pattern of differentiation does not appear to be related to taxonomic identity. However, because of the limited number of taxa involved, the analyses may be insensitive to the nuances of developmental variation among groups.

Relative timing and sequence of stages

Inter-specific differences in the sequences of developmental stages are also apparent in Figure 4.3. Recall first, however, that the sequence of stages is based on Defaure and Hubert's (1961) observations on *L. vivipara*. A smoothly ascending curve thus means the sequence of stages is the same as that exhibited by this species. Four, non-squamate species do not exhibit this pattern. The appearance of limb buds (DH stage 27) proceeds the appearance of the allantois (DH stage 25) during the development of the turtles *Chrysemys picta* and *Chelonia mydas*, and the fowl, *G. gallus*. For *A. mississippiensis*, the allantois and the limb buds appear at about the same time in development. By contrast, the allantois appears well before limb buds for the remaining species, all of which are lizards. If this pattern holds up after a more extensive survey of reptiles and birds, then the timing of these two developmental events may represent a phylogenetic distinction between squamates and other reptiles.

Developmental arrest

Development of reptilian embryos may be arrested temporarily. Arrest can be a facultative response to immediate environmental conditions. For example, development may cease when nest temperature drops below some critical level and resume when temperature rises above that level (Andrews *et al*., 1997; Chapter 5). While such cold torpor allows embryos to survive through short periods of inclement weather, periods of cold longer than a few days are associated with enhanced mortality (Christian *et al*., 1986). Many birds exhibit a parallel type of developmental arrest; development is arrested after oviposition because eggs are not brooded until the clutch is complete (Hébert, 2002). For birds, delayed brooding is a mechanism by which the parents synchronise development and hatching of

the eggs. The lengths of time that reptilian embryos can be exposed to temperatures too cold for development to occur without enhanced mortality are species specific and imply various grades of adaptation to variation in weather during incubation (Ewert, 1991). While cold torpor can occur any time during development, turtles exhibit several additional strategies for delaying emergence from the nest in response to inimical weather conditions. They can remain in the shell in a state of aestivation (Ewert, 1991). They can also remain in the nest for considerable periods after hatching if the soil is too dry or hard for hatchlings to dig an exit hole (Goode and Russell, 1968).

On the other hand, developmental arrest can be obligate part of the life cycle and occur regardless of environmental conditions. Several such types of developmental arrest are known for reptilian embryos, particularly for turtles (Ewert, 1991; Ewert and Wilson, 1996). Pre-ovipositional arrest is characteristic of all turtles studied to date; development becomes arrested within the oviducts when embryos are late gastrulae, and does not resume until after oviposition (Ewert, 1985). While oviposition normally occurs within weeks of fertilisation, egg retention, and thus developmental arrest, may be prolonged if suitable nesting conditions are not available. For example, although oviposition by most female *Deirochelys reticularia* turtles occurs in the autumn, some females retain eggs over winter and lay them the following spring (Buhlman *et al*., 1995). For the Australian turtle *Chelodina rugosa*, embryos do not resume development after oviposition because eggs are laid underwater in temporary ponds (Kennett *et al*., 1993). Embryos remain in a state of pre-ovipositional arrest until the pond dries and atmospheric oxygen diffuses into the egg.

In contrast to the universality of pre-ovipositional arrest in turtles, neither crocodilians nor birds normally exhibit this type of arrested development. In lepidosaurs, pre-ovipositional arrest occurs and may be widespread. Eggs of the tuatara (*Sphenodon punctatus*) are in the oviduct for 6-8 months before oviposition (Cree *et al*., 1992). Embryos are still only at the gastrula stage at oviposition (Moffat, 1985), so development is presumably arrested while eggs are in the oviduct. Chameleons that exhibit embryonic diapause (see below) may also exhibit pre-ovipositional arrest given that embryos are gastrulae at the time of oviposition.

For several of species of lizard, pre-ovipositional arrest occurs relatively late in development. If eggs of North American iguanids *Sceloporus undulatus* and *Urosaurus ornatus* are retained beyond the normal stage of oviposition because suitable oviposition sites are not available, embryonic development is arrested at DH stage 30 (Mathies and Andrews, 1999; Andrews and Mathies, 2000). Under similar circumstances, embryos of the Indian agamid *Calotes versicolor* become

arrested at stage 34 (Radder *et al*., 1998). Developmental arrest in these cases appears to be the result of insufficient oxygen for continued development (Andrews, 2002); once oviposition occurs normal development is resumed.

A difficulty in detecting developmental arrest is that embryogenesis may be arrested at stages when oviposition normally occurs. As a consequence, oviposited eggs always contain eggs of the proper stage, and eggs hatch in the normal amount of time. Arrest can only be detected by comparison of embryos in eggs laid at the normal time of oviposition (control) with embryos in eggs that are retained *in utero* for various lengths of time (experimental). Studies of this sort would provide the information needed to see if and when developmental arrest occurs. Currently, the response of embryos to extended egg retention has been examined for only a few squamate species (Andrews and Mathies, 2000), but this type of developmental arrest could prove to be relatively common. It is clear that more research is needed for a wider range of species.

A type of post-ovipositional arrest that occurs after oviposition is embryonic diapause, i.e. 'arrested development when the immediate proximate environment would normally foster active development' (Ewert, 1991). Embryonic diapause is known only for a few species of turtles and chameleons, and only occurs at the gastrula stage. Turtles with known or suspected (from lengthy periods of incubation) embryonic diapause are members of the families Chelidae, Kinosternidae, Emydidae, Testudinidae, and Trionychidae (Ewert, 1991). The common denominators of embryonic diapause are pronounced seasonality and warm temperate to tropical climates. In warm temperature regions, diapause is associated with oviposition in the autumn. Development remains arrested until spring. A period of cool temperature, which would occur normally during winter, appears to be necessary to break diapause, as eggs that are not cooled may never resume development (Ewert and Wilson, 1996). For the turtle *Kinosternon baurii* in Florida, the tendency for eggs to diapause is season specific. For example, eggs laid in the spring initiate development immediately and hatch in the autumn of the same year. Eggs laid in the autumn go into diapause and hatch in the autumn of the following year (Ewert and Wilson, 1996). The turtle *Chelodina expansa* of southern temperate Australia also lays its eggs in the autumn, and hatchlings also emerge from the nest roughly a year later. Embryos exhibit both a continuation of the pre-ovipositional arrest and then enter period of post-ovipositional diapause after oviposition (Goode and Russell, 1968; Booth, 1998c, 2000b, 2002a). While *C. expansa* exhibit diapause and a lengthy incubation period, two other species of turtle at the same site exhibited more typical reproductive cycles with oviposition in the spring and hatching during summer and autumn (Goode and Russell, 1968). Indeed, species with diapause typically occur at sites with non-diapausing species.

The diversity of turtle reproductive strategies at any one site is thus likely to reflect both the past history of the species as well their adaptation to their present environments (Kennett, 1999).

In tropical and sub-tropical regions, diapause is associated with seasonality of rainfall rather than temperature (Ewert, 1991). In tropical northern Australia, for example, female *Elseya dentata* turtles nest in the wet season with hatching occurring the following wet season; the initial part of the roughly six month incubation period appears to be spend in diapause (Kennett, 1999). Diapause is also characteristic of species of *Kinosternon*, *Staurotypus*, and *Rhinoclemmys* with ranges in Pacific coastal regions of Mexico and Central America (Ewert and Wilson, 1996), areas that exhibit distinct wet-dry seasonality but little annual variation in temperature.

Embryonic diapause in chameleons has been documented by direct observations on embryos of a few species in the subgenus *Chamaeleo* and the genus *Furcifer* (Bons and Bons, 1960; Blanc, 1970), and is inferred from the long incubation periods of other species in these taxa (Necas, 1999). Incubation periods of six months to a year or more (Ferguson, 1994; Necas, 1999) are likely to include a period of diapause; incubation periods of two to three months are typical of lizards that lay eggs of a similar mass (1–1.5 g) of chameleon eggs (Birchard and Marcellini, 1996). During diapause, chameleon embryos remain at the gastrula stage for several months or more after oviposition. For example, the average length of diapause for embryos of *Chamaeleo calyptratus* is 70 days at an incubation temperature of 28°C (Andrews and Donoghue, 2004).

Why does diapause characterise these particular groups of chameleons? Eggs of the temperate and sub-tropical *Chamaeleo chamaeleon* and *Chamaeleo zeylanicus* are laid in the autumn and hatching occurs the following summer or autumn and so the embryos are in diapause during winter months (Bons and Bons, 1960; Minton, 1966; Cuadrado and Loman, 1999). Eggs of the tropical *Furcifer lateralis* and *Furcifer pardalis* are laid during the wet (warm) season and hatching does not occur until the following wet season; so embryos are in diapause during the dry (cool) season (Blanc, 1970; Bourgat, 1970; Schmidt, 1986).

Diapause may reflect adaptation to climatic seasonality if diapausing gastrulae are better able to withstand cool or dry conditions than more developed embryos. On the other hand, diapause may be less related to the development of the embryo *per se* than to the time when the eggs hatch. Embryonic diapause would thus serve as mechanism that positions hatching at a time most suitable for the survival of neonates when they leave the nest. Eggs of varanid lizards invariably hatch during the wet season at the time of maximum abundance of prey for neonates,

and the lengthy incubation periods of most species (Phillips and Millar, 1998) suggest that a period of diapause insures hatching at the appropriate time.

Freshwater turtles in the temperate zone exhibit another strategy for controlling the time of emergence. Individuals complete their development during the summer, but hatchlings remain in the nest over winter (Gibbons and Nelson, 1978). Over-wintering in the nest could be considered a form of hatchling diapause (that grades into cold torpor during the winter) as individuals remain in the nest in the autumn even when ambient physical conditions are suitable for emergence. Some species, however, such as *Sternotherus odoratus*, exhibit a mixed strategy; some hatchlings emerge from the nest in the autumn (Mitchell, 1988) or the following spring, or both (Gibbons and Nelson, 1978).

Remaining in the nest over winter poses a serious physiological challenge for hatchlings of species that live at latitudes where nest temperature drops substantially below freezing. Research conducted in the early 1990s suggested that hatchlings of the painted turtle *C. picta* survive exposure to low temperature in nests because they are tolerant of freezing (Churchill and Storey, 1992). The problem with this theory is that freeze tolerance of hatchling turtles is actually limited to quite high sub-zero temperatures. For example, while hatchlings of a few species can survive freezing at –2°C for short periods, lower temperatures are fatal (Packard *et al*., 1999). However, hatchlings survive exposure to much lower temperatures in nests during the winter, and so some mechanism other than freeze tolerance must be operating in nature. This mechanism is supercooling (Packard *et al*., 1997, 1999; Costanzo *et al*., 2000, 2001; Packard and Packard, 2001). Hatchling *C. picta* are able to supercool for two reasons. One is that their body fluids do not contain suitable motes (organising sites) where molecules of water can condense and form crystals of ice. Secondly, the epidermis of the head and feet contains a lipid layer that resists penetration of ice crystals that grow inward from the frozen soil. When eggs hatch in the fall, however, hatchlings have a limited ability to supercool; seasonal acclimation to declining temperatures after hatching is associated with an increased capacity to supercool and also to resist inoculative freezing (Costanzo *et al*., 2001). While hatchlings retain the ability to move their head and limbs even at sub-zero temperatures (Costanzo *et al*., 1999), it is not clear if such activity in the nest would provide any benefit to hatchlings as ambient temperature fluctuates during the winter months. These features allow hatchlings of *C. picta* to avoid freezing at nest temperatures as low as –10°C or even lower.

Hatchling emergence

Hatching

The last embryonic, or perhaps the first neonatal, action is hatching. A tooth or

tooth-like structure on the tip of the upper jaw or beak facilitates rupture of embryonic and shell membranes. For birds, crocodilians, turtles, and tuatara, this structure (caruncle) is not a true tooth as it develops as a horny outgrowth at the midline of the upper jaw. For squamate reptiles, the structure (egg tooth) is a true dentinal tooth on the premaxilla (Lillie, 1952). Regardless of embryological origin, however, caruncles and egg teeth are functionally identical. At term, bird embryos use stereotyped sets of behaviours to break the shell (Bond *et al*., 1988). These include striking the shell with the beak while simultaneously rotating their body so that the shell is pipped in a ring or arc at one end of the egg and using the body and legs to put pressure on the shell. The behaviour of reptile embryos at hatching is largely unknown but probably parallels what is known about bird embryos. When shells are parchment-like, embryos slit (or tear) one end of the shell in several to many wide arcs using the egg tooth or caruncle. The claws on the forelimbs of turtles may help tear open the shell (Ewert, 1985). For *A. mississippiensis*, microbial degradation and calcium removal weaken the heavily mineralised shell and facilitate rupture of the shell membranes by the term embryo (Ferguson, 1985) although this is not a pre-requisite for hatching as eggs incubated without substrate have no difficulty in hatching (Deeming, personal communication).

In reptiles, and in megapode birds (Booth and Jones, 2002), breaking of the eggshell at hatching releases a considerable amount of fluid. This is allantoic fluid (although sometimes attributed as being albumen, e.g. Badham, 1971) the volume of which is correlated with the fluid uptake by the egg (Deeming, 1988), or in the case of *A. mississippiensis*, the degree of weight lost from the egg (Deeming and Ferguson, 1989a). Fluid within the egg appears necessary for successful hatching. If the 'excess' fluid drains from the egg and the shell membranes dry, hatchlings can become trapped in the shell membranes and die.

The transition from an embryo living within the confines of an egg to a mobile neonate ranges from several hours to days. Turtles typically pip the shell such that the head protrudes from the egg, and they remain partially within the shell for several days. Crocodilians depart from the egg relatively quickly, within a few minutes to within a day of pipping (Ferguson, 1985). Squamates also tend to leave the egg relatively quickly; once the egg is pipped hatchlings may emerge extremely rapidly if the nest or egg is disturbed. No comprehensive set of observations has been made to determine the reason for intra- and interspecific variation in the length of the period between pipping and exiting the egg. For turtles in particular, this period is associated with the absorption of residual yolk into the gut and the unfolding of the carapace and plastron (Ewert, 1979, 1985). For other reptiles, the yolk may be absorbed prior to pipping, and in general residual

yolk serves as source of energy for neonates for their first few days to weeks of life. Some snakes leave a portion of their yolk sac in the egg at hatching following low temperature incubation (Burger *et al*., 1987) or fungal infection (Deeming, 1988). It is unknown to what extent this ability extends to other taxa. Other reasons for delayed departure from the egg include withdrawal of blood from the CAM and the physiological transition to pulmonary respiratory.

Synchronisation of hatching and emergence from the nest

For reptiles, hatching and emergence from the nest can be more or less synchronous or be separated by days, weeks, or even months. These events will therefore be considered separately. Reptiles typically ovulate multiple eggs simultaneously (Chapter 3) and the development of embryos is essentially synchronous within a clutch and hatching is more or less synchronous as well. Environmental conditions, however, can create gradients of temperature, moisture, and gas concentrations within a nest (Chapters 2 and 5) and thus hatching within a clutch can spread over several days or more. Such gradients in physical conditions within the large clutches of sea turtles are well documented (Booth and Thompson, 1991).

Some observations suggest that communication among clutch mates can act to synchronise hatching such eggs in close proximity to one another hatch simultaneously while isolated eggs from the same clutch hatch over an extended period of time (e.g. Annis, 1995). The communication mechanism may simply be the physical jostling of eggs placed in close proximity in nests. Physical disturbance certainly prompts hatching. For example, when a nest of the lizard *Plica plica* was uncovered and the eggs disturbed, the five eggs hatched simultaneously and the hatchlings fled (Vitt, 1991). In birds (Brua, 2002) and crocodilians, however, embryo-embryo communication is important in hatching synchrony. Crocodilian hatchlings commonly call from the nest in order to alert their mother to their impending hatch and so facilitate the opening of the nest and transport to water (Ferguson, 1985). This calling takes place within intact eggs that contain significant volume of allantoic fluid in a sac that surrounds the hatchling. How can the hatchling fill its lungs with air in this situation? Careful examination of near term *A. mississippiensis* eggs (n = 10) has revealed air within the amniotic sac (Figure 4.4; Deeming and Ferguson, unpublished observations). Clearly, this allows the respiratory system to function normally and allow calling but it is not known how the air traverses the eggshell to enter the amnion.

Hatchlings typically emerge from the nest at the same time and such synchronisation can occur because hatching is more or less simultaneous or because departure from the nest is delayed until all eggs have hatched. The latter is certainly the case for those turtles that overwinter in the nest and that emerge the following

Figure 4.4. Full term embryo of *Alligator mississippiensis* with the upper hemisphere of eggshell and chorio-allantois removed to reveal, as indicated by the arrow, an amniotic sac full of air around the head of the embryo (Deeming and Ferguson, unpublished observations).

spring. What is the benefit of synchronised emergence? One obvious benefit is to ensure physical escape from the nest. For clutches that are buried deeply or that are in soils that have dried or compacted during incubation, the efforts of a single hatchling might not be sufficient to dig from the nest to the surface. The digging activities of several hatchlings would be more effective than one. For some species of varanid lizards and crocodilians, however, eggs are typically deposited in nests from which escape of hatchlings is difficult or even impossible. In these cases, a parent or parents return to the nest site and open the nest allowing the hatchlings to escape (see Shine, 1988 for a description of this and other types of parental behaviour).

Synchronised departure from the nest may also serve to reduce predation on the clutch. For example, hatchling green iguanas (*I. iguana*) scan the nesting area from the nest opening before departing from the nest in groups. Departure from one nest is usually synchronised with departures from other nests as well. Individuals leaving the nest en mass may be more successful in both avoiding predators and in confusing predators if they are attacked than single individuals (Burghardt *et al*., 1977). Synchronised departure from the nest seems unlikely to serve as a predator satiation strategy. The relatively large predators that would be attracted to hatchlings would be able to eat multiple hatchlings before satiation. It seems more likely that predator satiation is a female nesting strategy by which simultaneous emergence of hatchlings from many nests allows more hatchlings to escape than if females nested in isolation.

Factors affecting hatchling size

In general, large species produce large hatchlings and small species produce small

hatchlings. The exact relationship differs among snakes, lizards, turtles, and crocodilians, but for all groups, hatchling size is a relatively smaller proportion of adult size as adult size increases (Andrews, 1982). Not surprisingly, hatchling size is associated with egg size. For crocodilians and turtles with rigid shelled eggs, hatchling mass is a constant proportion of egg mass at oviposition (Deeming and Ferguson, 1991b). This is not true for flexible-shelled eggs of pliable-shelled turtles and squamates where as initial egg mass increases the hatchling is a smaller proportion of egg mass (Deeming and Ferguson, 1991b). At any adult size, interspecific variation in hatchling size is related, in part, to particular life history strategies (Andrews, 1982).

Intraspecific variation in hatchling size can also be substantial. The size of the hatchling is affected by factors unique to the individual (its genes), intrinsic to the egg (maternal effects), extrinsic to the egg (maternal environment prior to oviposition and the physical environment after oviposition), and by the interaction of those factors (gene-environment interactions). Of course, many phenotypic traits of hatchlings are affected the environment during incubation, such affects and their relationship to fitness are discussed in Chapter 10. In this section the focus is on genetic, maternal, and environmental effects on hatchling size. Hatchling size *per se* is an important life history variable in its own right. While bigger hatchlings may be fitter than smaller hatchlings (e.g. Packard, 1999), hatchling size is part of an important trade-off for female fitness. The investment in offspring involves a finite amount of energy, and so selection for larger offspring will reduce the total number of offspring produced (Lack, 1954). As a consequence, the biggest hatchlings may not always optimise fitness for a given female and ecological circumstance (Sinervo and Doughty, 1996). Given the conceptual importance of hatchling size to life history theory and to the wealth of observations of strictly environmental influences on hatchling size, a general review of the diverse factors that affect hatchling size seems appropriate.

A genetic component to hatchling size is implicated by significant clutch effects in studies that include clutch as a class effect in the experimental design (e.g. Phillips and Packard, 1994; Packard *et al*., 1998; see also Chapter 10). To distinguish between treatment and clutch effects, Warner and Andrews (2002b) for example, analysed their data with a two-way ANCOVA. Moisture level during incubation was one main effect, clutch was the other, and initial egg mass was used as a covariate to factor out one maternal effect, i.e. the amount of provisioning of the egg. Results of this study were typical, with both treatment and clutch affected hatchling size. Significant clutch effects, however, do not demonstrate unequivocally that variation among clutches includes a genetic component; variation among clutches could consist entirely of maternal effects. This ambiguity

of clutch effects was neatly circumvented in a study of genetic determinants of hatchling size in the sand lizard *Lacerta agilis*. Olsson *et al*., (1996) used the combination of a genetic marker and DNA finger printing to assign paternity to half-sibs (same mother, different fathers). Clutch effects on hatchling size were stronger than paternal effects, as expected, but paternity did affect hatchling body size, thus isolating a genetic component to variation in hatchling size (males contribute only genes). By determining mother-daughter correlations in egg and hatchling size, Sinervo and Doughty (1996) were also able to identify genetic effects on hatchling size. In their study, significant mother–daughter correlations persisted even when yolk was removed from eggs to produce relatively small hatchlings (daughters).

Maternal effects on hatchling size include a wide range of phenomena including the amount of investment in the egg. This type of maternal effect so common that investigators typically account for the positive association between egg size and hatchling size by using initial egg mass as a covariate in statistical analyses (e.g. Lesham *et al*., 1991; Phillips and Packard, 1994; Ji and Du, 2001b). The amount of investment per egg is related to environmental conditions and thus nutrient availability for females. For example, hatchlings of the sand lizard *L. agilis* were larger on average in years when females were in better condition and weather conditions were more favourable to foraging than in years when females were in poorer condition (Olsson and Shine, 1997a). In addition, hatchling size was negatively related to the size of the clutch relative to the body size of females (but not to the body size of females *per se*). This negative relationship occurred in all five years of the study regardless of the mean size of hatchlings *per se*.

Other types of maternal effects include the thermal behaviour of females prior to oviposition or during incubation. Given that incubation temperature affects hatchling phenotypes (see below), thermal behaviour of the female prior to oviposition or behavioural manipulations of the nest or eggs have the potential to affect hatchling size (see Chapter 8). In addition, the quality of maternal provision in terms of nutrients *per se*, the balance of nutrients, or hormonal deposition in the yolk also have the potential to affect hatchling size. For example, the amount of testosterone deposited in the eggs of the lizard *Anolis carolinensis* exhibits strong inter-individual variation and could affect size, or other phenotypic traits (Lovern and Wade, 2001). For some chelonians, female size may actually constrain egg size (and thus hatchling size) because the size of the egg is limited by the size of the pelvic opening (Congdon and Gibbons, 1987).

The physical conditions experienced by the egg during incubation also affect hatchling size. Temperature and moisture are the most general players in this regard.

Field studies in which temperature or moisture or both were monitored during incubation document variation in hatchling size that is associated with variation in these physical factors (Snell and Tracy 1985; Cagle *et al*., 1993; Shine *et al*., 1997b; Packard *et al*., 1999). Gaseous conditions in the nest can also affect hatching phenotypes, but such effects appear to be special cases within reptiles (see Chapter 10).

The results of controlled laboratory studies and biophysical modeling indicate that temperature and moisture affect hatchling size through their joint influences on metabolism of the embryo and the water balance of the egg (Ackerman *et al*., 1985a; Packard and Packard, 1988a; Packard, 1991; Ackerman, 1994). These affects have been amply documented for studies on the eggs of turtles. In general, eggs incubated at low temperatures and under moist conditions produce relative large hatchlings with small amount of residual yolk and eggs incubated at high temperatures and dry conditions produce hatchlings that that are relatively small and have large amounts of residual yolk (Packard *et al*., 1987; Packard, 1999). This pattern is the result of the interaction between temperature and moisture on the water dynamics of eggs. While eggs absorb more water under moist than dry conditions, water uptake declines with an increase in temperature because of the enhanced metabolism of the embryo increases the temperature of the egg relative to the environment. Even small increases in the temperature of the egg result in the evaporation of water from the egg. This effect is particularly pronounced late in development as the embryo increases rapidly in size and metabolic rate. The conversion of yolk nutrients into embryonic tissue is thus affected by the amount water uptake by eggs, which is determined by both moisture and temperature. Data on *C. johnstoni* hatchlings clearly illustrate effect of temperature on the utilisation of yolk by embryos (Table 4.4); residual yolk mass increases with incubation temperature in accord with a decline in hatchling mass whether measured as a whole or as yolk-free mass (Webb *et al*., 1987c).

Table 4.4. Effect of incubation temperature on the composition of *Crocodylus johnstoni* hatchlings. Data from Webb *et al*. (1987c).

Temperature (ºC)	*Residual yolk mass (g)*	*Yolk-free hatchling mass (g)*	*Live hatchling mass (g)*
28.0	4.5	40.4	44.8
29.0	5.8	38.5	44.3
30.0	7.1	36.6	43.7
31.0	8.4	34.7	43.1
32.0	9.7	32.8	42.5
33.0	11.0	30.8	42.0
34.0	12.3	28.9	41.4

Studies on turtles most clearly illustrate how temperature and moisture affect hatchling size. Variation in these factors, however, does not always affect hatchling size. For example, the snout-vent length of *S. undulatus* hatchlings increases with incubation temperature (Andrews *et al*., 2000) in contrast to the general prediction that size should decrease. Moreover, variation in temperature did not affect the size of hatchling *A. mississippiensis* (Allsteadt and Lang, 1995; Congdon *et al.*, 1995), the soft-shelled turtle *Trionyx triunguis* (Leshem *et al.*, 1991), and the river turtle *Emydura signata* (Booth, 1998c). At the same range of temperature and moisture (27–28 to 31°C and –150 to –1100kPa), both factors affected the size of hatchling white-throated savannah monitors *Varanus albigularis* (Phillips and Packard, 1994) but did not affect the size of hatchling Cuban rock iguanas *Cyclura nubila* (Alberts *et al*., 1997).

In their review of the literature, Flatt *et al*. (2001) suggest that variation in the sensitivity of embryos to environmental conditions may be an adaptive response that reflects characteristics of normal nest sites. They further argue that species that place their eggs in deep nests that are relatively buffered from changes in moisture may be more sensitive to variation in moisture than species that place their eggs in shallow nests where some level of buffering is important for survival and observe that temperatures may affect hatchling size more than moisture (Flatt *et al.*, 2001; Ji and Du, 2001b). Taxa specific features such as nest depth and substrate, egg size, and shell type may thus explain the divergent conclusions of Packard (1999) and Flatt *et al.* (2001) on the relative importance of temperature and moisture on hatchling size.

This idea is supported by observations on turtles. For example, the amount of variation in hatchling size of snapping turtles (*C. serpentina*) explained by nest temperature and moisture was comparable, but relatively low at a site in Nebraska (Packard *et al.*, 1999). At the same site, more variation in hatchling size of painted turtles (*C. picta*) was explained by moisture availability than temperature during incubation (Cagle *et al*., 1993). Packard *et al*. (1999) concluded that differences in the results of the two studies are because nests of snapping turtles are relatively deep and relatively buffered from environmental variation than the shallower nests of painted turtles.

Future directions for research into embryonic development in reptiles

This review supports prior assessments that the general patterns of reptilian and avian development are highly conservative. Incubation temperature accounts for much of the variation in the overall rate of development and the timing of

differentiation relative to the total length of the developmental period. Despite congruence of broad patterns of development, biologically significant variation among taxa may exist. At present, however, it is impossible to evaluate the existence of, let alone the importance of, variation in developmental patterns. Broadly comparative data on reptilian development are woefully lacking. For example, the allantois appears prior to the appearance of the limb buds in lizards, but at about the same time or later on birds, crocodilians, and turtles. Is this distinction an artefact of the small sample of taxa? Or, is variation biologically meaningful? If so, does the relatively early appearance of the allantois in lizards reflect oviposition relatively late in development and a functional need for enhanced oxygen exchange by well developed embryos within the oviduct? Or, is the time of appearance of the allantois simply related to neutral variation established early in the phylogeny of reptiles? These questions underscore the lack of comparative information on reptilian development. In particular, studies on morphological and functional association between the development of the extra-embryonic membranes and their associated fluid compartments and the differentiation and growth of the embryo would be of particular importance. This and other comparative studies could provide insights into the developmental events that are most highly constrained and those that have been free to vary among taxa.

A second area that could be profitably targeted for study concerns temperature sensitive periods of development. An abundance of research documents that variation in phenotype is associated with variation in temperature during incubation (Chapter 10). Whether such temperature effects are temporary or continue far in post-hatching life, they have the potential to affect fitness. The problem is that temperature sensitive periods are known only for sex determination. For example, manipulations of incubation temperature demonstrate that sex of turtles with temperature dependent sex determination (TSD) is determined during the middle-third of development, a period corresponding to the differentiation of gonadal tissue (Bull and Vogt, 1981; see also Chapter 9). Sex, however, is the only phenotypic trait for which the critical stages for temperature sensitivity are known. The TSD model suggests that temperature could affect other phenotypic traits at specific stages during development. If correct, determining when during development this influence occurs is important for two reasons. First, the interplay between temperature and phenotype is of intrinsic interest. For example, is morphology and performance (e.g. limb size and proportions, running speed, *etc*.) affected by temperature during organogenesis when the basic form and components of an organ or structure are formed or during late development, when increase in size of organs and structures characterises development? Or, alternatively, is the effect of temperature on morphology and performance of an individual so integrated across development that temperature sensitivity is not an issue? The second reason

for determining when temperature affects phenotypic traits is to enhance experimental studies. Knowing the period of temperature sensitivity would mean that the onset of temperature manipulations could be tailored to the sensitive stages for particular traits. Such information would also facilitate interpretation of field studies where short-term fluctuations of temperature are the norm and where temperature patterns can shift during the incubation period (Shine, 2002b).

In conclusion, opening the 'white box' can provide novel opportunities and insights for research on a wide range of topics directly or indirectly related to development. The overriding impact of temperature on reptilian development suggests that accounting for temperature is a crucial element to conducting and interpreting comparative studies on embryonic development. Finally, the benefits of incorporating embryos into experimental designs include: 1) the availability of good staging tables for lizards, crocodilians, and turtles, 2) one gravid female typically provides several to many eggs, 3) development is synchronised within a clutch, and 4) eggs from each clutch can be allocated into different experimental treatments providing experimental replication with a known starting point in development.

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