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The influence of temperature on embryonic developmental arrest in marine and freshwater turtles

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ABSTRACT

Temporary arrest of embryonic development can occur both pre- and post-oviposition in turtles. Pre-ovipositional arrest is an obligate part of the life cycle and occurs universally in turtle embryos, commencing while eggs are in the oviduct and persisting until after oviposition. Pre-ovipositional arrest allows turtle mothers the flexibility to choose an optimum time to nest and provides embryos some capacity to respond to varying environmental conditions immediately after eggs are laid. Following oviposition, turtle embryos are known to be significantly affected by incubation conditions and specifically, temperature has a profound influence on developmental rate and success of embryos. We conducted a comparative investigation of how temperature influences (1) the duration of pre-ovipositional arrest after eggs are laid, (2) the number of embryos that fail to recommence development and (3) hatching success, using eggs of the green sea turtle (*Chelonia mydas*), and three species of freshwater turtle; the western oblong turtle (*Chelodina oblonga*), the eastern longneck turtle (*Chelodina longicollis*), and the Murray River turtle (*Emydura macquarii*). We incubated arrested eggs of each species at three different temperatures (low, medium, high) and monitored embryonic development immediately after oviposition and throughout incubation. Interspecific variation was evident in the effects that temperature had on pre-ovipositional arrest, subsequent embryonic development and hatching success. A major finding of this study was that, with the exception of *E. macquarii*, there was no significant difference in the time to white spot development (the first external visible sign of embryological development following arrest) between temperature treatments, suggesting that the resumption of development and the breaking of pre-ovipositional arrest after eggs are laid are independent of temperature. Furthermore, although the number of *C. mydas* eggs to successfully recommence development after oviposition was consistently high (~97–100%) across the three temperature treatments, a significant proportion of *C. oblonga* and *E. macquarii* eggs failed to resume development. In both the low and high temperature treatments the rate of *C. oblonga* embryo mortality was 95% and 60%, respectively, and for *E. macquarii* it was 53% and 24% respectively. These findings bring us a step closer to understanding why failure to recommence development after oviposition causes high rates of early stage embryo mortality and decreased hatching success in turtles.

1. Introduction

Temporary arrest of embryonic development can occur both pre- and post-oviposition in turtles (Ewert, 1985, 1991). Pre-ovipositional arrest is an obligate part of the life cycle, occurring universally when embryos are gastrulae. Pre-ovipositional arrest begins approximately one week after fertilisation while eggs are in the oviduct and persists until oviposition, regardless of the duration of egg retention (Miller, 1985). Oviducal hypoxia maintains arrest *in utero* with resumption of development occurring upon exposure to atmospheric oxygen levels when eggs are laid (Rafferty et al., 2013). This form of arrest is a pivotal part of the life cycle in turtles and marks the transition from a maternal to nest environment. Failure to recommence development after oviposition is a major cause of mortality in the critically endangered leatherback turtle, contributing largely to low hatching success in this species (Bell et al., 2004; Rafferty et al., 2011).

In contrast, post-ovipositional arrest is quite rare in turtles and has only been documented in a limited number of freshwater species. Post-ovipositional arrest can be categorised into two obligatory groups based on the environmental stimulus involved, the first of which being a hypoxia induced extension of pre-ovipositional arrest, known only in the northern long-necked turtle (*Chelodina rugosa*) (Kennett et al., 1993). *C. rugosa* lay their eggs in underwater nests during the rainy season in Northern Territory, Australia, and embryonic development only resumes when the nest dries out and atmospheric oxygen penetrates the eggshell (Kennett et al., 1993).

The second is a temperature induced embryonic diapause (ED) observed after white spot formation (the first external visible sign of embryological development following arrest), prior to somite and vitelline development (Booth, 2002a; Ewert and Wilson, 1996). ED of

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healthy embryos occurs in an environment that normally promotes active development, yet without an obligatory trigger to re-start development the embryos remain arrested and eventually die (Ewert, 1991). In turtles, this trigger is generally a period of chilling, usually during winter (Andrews et al., 2008; Booth, 2002a; Ewert and Wilson, 1996). Currently, 33 species of freshwater turtle are known to express ED and Horne (2007) estimates that a further 63 unstudied species are also likely to express it (Horne, 2007). Both known and suspected species have protracted incubation periods and are members of the families Chelidae, Kinosternidae, Emydidae, Testudinidae, and Trionychidae (Ewert, 1991; Horne, 2007).

Developmental arrest can also occur in mature embryos and is known as embryonic aestivation. Embryonic aestivation prolongs the residence of a fully formed embryo within an unpipped egg for weeks or months if environmental conditions are *un*favourable (Ewert, 1991; Ewert and Wilson, 1996). Similar to ED, a trigger is needed to stimulate pipping and hatching and in its absence embryos become weak and eventually die if residual energy reserves become depleted (Ewert, 1991; Horne, 2007). Hypoxia is the trigger to initiate hatching of pignose turtle (*Carettochelys insculpta*) eggs, induced by nest inundation during heavy rains and flooding (Doody, 2011; Doody et al., 2001).

Evidently, temperature and oxygen availability play a primary role in the onset, maintenance and breaking of each type of developmental arrest in turtles. However, our knowledge of each process is restricted to a limited number of species and substantial investigation remains to be conducted. Timely research on the topic is also particularly pertinent considering that anthropogenic global warming may result in an elevation of global mean air temperature by 1–4.5 °C by the year 2100 (IPCC, 2007). Global warming is predicted to decrease leatherback turtle hatching and emergence success (Saba et al., 2012) and possibly influence marine turtle hatchling sex ratios (Poloczanska et al., 2009), thus highlighting the potential impact that temperature fluctuations may have on turtle embryonic development.

The primary objective of this study was to test the comparative effects of temperature on developmental arrest in two turtle families (Cheloniidae and Chelidae). We achieved this by observing embryonic development in eggs of the green sea turtle (*Chelonia mydas*), and 3 species of freshwater turtle; the western oblong turtle (*Chelodina oblonga*), the eastern longneck turtle (*Chelodina longicollis*) and the Murray River turtle (*Emydura macquarii*). Species were chosen based on their differences in geographical range, eggshell type, nesting habitat and incubation duration; variables predicted to influence developmental arrest in turtles (Horne, 2007). All four species are known to undergo pre-ovipositional arrest yet the effects of temperature on both the duration of arrest after eggs are laid and the success rate of embryos remains to be examined. Further, both *C. longicollis* and *C. oblonga* are estimated to have an almost 70% likelihood of expressing ED but no studies to verify this have been done (Horne, 2007).

To achieve our primary objective we incubated eggs at three constant temperatures from the time of oviposition until either hatching occurred or eggs were determined to be dead. Temperature treatments were chosen to reflect the range of temperatures (low, medium, and high) experienced in natural nests of each species. First, we evaluated the response of embryos in a state of pre-ovipositional arrest to differences in temperature immediately after oviposition. As mentioned earlier, all turtle embryos are laid early in the development schedule as arrested gastrulae (Ewert, 1985; Miller, 1985), allowing comparisons of development to be easily made between temperature treatments. We then assessed whether each species expressed other forms of developmental arrest in response to temperature by looking at the developmental stages of embryonic mortality at each temperature. We anticipated that mortality would be pronounced at specific stages (after white spot formation but prior to somite and vitelline development, and prior to pipping in fully formed embryos) because at constant temperatures arrested embryos would die in the absence of a temperature change (environmental trigger to recommence development).

2. Materials and methods

2.1. Study species

C. mydas has a circumglobal distribution with nesting occurring in over 80 countries worldwide following significant trans-continental migrations (Hirth, 1997). In northern Queensland, Australia, mating is observed between September and November (Limpus, 1993), with nesting occurring between October and March (Bustard, 1972). On average, females nest every 5 years or so, during which they will typically lay approximately 5–6 clutches every two weeks (Limpus et al., 1994). The mean egg incubation period in this region is about 65 days (Limpus, 2008), somewhat similar to that observed in *E. macquarii* nests (Goode and Russell, 1968).

C. oblonga is confined to south-west Western Australia and mating takes place during late winter and spring with nesting occurring between September and January. This species generally lays up to three clutches of up to 16 eggs each per annum (Kuchling, 1988, 1989). Egg incubation takes approximately 210–220 days under natural conditions (Clay, 1981), although it may be considerably extended, taking up to 291 days (MCCutcheon, 1985).

C. longicollis ranges from eastern South Australia, throughout Victoria and most of New South Wales and into eastern Queensland (Cogger, 1992). *C. longicollis* typically mate in September and nest between October and January, laying up to three clutches annually that can consist of up to 23 eggs per clutch (Kennett et al., 2009). Incubation generally takes between 110 and 150 days with hatchlings emerging in autumn in most instances, although hatchlings may overwinter in the nest in some locations (Kennett et al., 2009).

E. macquarii shares a similar distribution pattern to that described for *C. longicollis*, although mating in this species is usually observed between March and April (Cann, 1998). Nesting then takes place between October and December, during which females produce a single clutch of up to 35 eggs (Chessman, 1986; Spencer, 2001). In natural nests, the average incubation period described for this species is 75 days (Goode and Russell, 1968). Interestingly, eggs of *E. macquarii* that are deposited at the same time of year, and under the same conditions as *C. longicollis*, have much shorter incubation periods than *C. longicollis*.

2.2. Study locations

Gravid *C. oblonga* were trapped from Lake Goolelall in Western Australia, between October 1st and 7th, 2010, using baited, modified funnel traps, while gravid *C. longicollis* and *E. macquarii* were trapped from Lake Coranderrk, Victoria, between October 10th and December 15th, 2010, using baited fyke nets. Gravid freshwater females were identified through manual palpation of the inguinal pocket. Those determined to be gravid using this method were later radiographed to confirm egg presence. All gravid freshwater females were induced to lay in the laboratory using an intramuscular injection of synthetic oxytocin (Butocin, Bumac Pty Ltd., NSW, Australia) at a dose of 15 IU/kg (Ewert and Legler, 1978), with oviposition generally occurring within 20 min. *C. mydas* eggs were collected from two females during oviposition on Heron Island, Australia, in December, 2010. All animals and resulting hatchlings were released alive at their point of origin at the conclusion of this investigation.

2.3. The breaking of pre-ovipositional arrest

Upon oviposition, all eggs were patted dry with a paper towel and marked on the shell for identification using a graphite pencil. *C. mydas* eggs that were collected in the field were transferred immediately to a refrigerator and cooled for 10 h at < 10 °C to halt embryonic development