# Maternal Effects in the Green Turtle (*Chelonia mydas*)

SUBMITTED BY SAM B. WEBER TO THE UNIVERSITY OF EXETER
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I certify that all material in this thesis which is not my own work has been identified, and that no material has previously been submitted and approved for the award of a degree by this, or any other university.

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**Green turtle covering a clutch of eggs on Long Beach, Ascension Island, UK.**

# Abstract

In oviparous animals, maternal traits such as the investment of resources in eggs and oviposition site selection are often important determinants of offspring phenotypic quality, and may have an adaptive role in tailoring offspring phenotypes to local environmental conditions. This thesis examines the adaptive significance of two specific maternal traits in the green turtle (*Chelonia mydas*); namely the deposition of fat-soluble antioxidants in egg yolk, and the selection of nest sites via natal homing. Diet-derived, fat soluble antioxidants, such as vitamin E and carotenoids, are ubiquitous components in the eggs of oviparous vertebrates, and are thought to have an adaptive role in buffering embryos and neonates against free-radical induced oxidative stress. However, evidence for such a function in wild populations is lacking. This thesis investigates the proximate sources of variation in yolk antioxidant concentrations in the green turtle, particularly in relation to maternal diet, plasma concentrations and laying sequence, and assesses the functional consequences of such variation for offspring phenotypes. Overall, the results presented suggest that maternal access to dietary antioxidants may be a relatively minor source of variation in egg concentrations in wild populations, and that independent physiological mechanisms may instead regulate the deposition of vitamin E and carotenoids in eggs. However, yolk concentrations of vitamin E and carotenoids did not influence offspring resistance to oxidative stress, and were not tailored to the offspring developmental environment. This was despite evidence that the maternally-provided nest environment strongly influenced offspring exposure to oxidative stress. Taken together, these results question the view that maternal deposition of fat-soluble antioxidants in eggs is an adaptive maternal effect to compensate for the risk of oxidative stress in offspring. Secondly, I investigated the adaptive significance of reproductive homing behaviour in green turtles. Female sea turtles generally return to nest at the particular site where they themselves were born (‘natal homing’), meaning that the offspring developmental environment may closely resemble that experienced by the mother. I therefore tested the hypothesis that natal homing facilitates the adaptation of developmental tolerances to specific environmental regimes. Using a common-garden rearing experiment I show that the offspring of females nesting on a naturally hot beach have markedly improved viability and growth at high incubation temperatures compared to the offspring of females from a nearby cooler beach. This disparity was not related to maternal provisioning of antioxidants or other key resources in eggs. These results suggest that natal homing may significantly increase maternal and offspring fitness by maintaining a stable selective environment across generations for the evolution of key fitness traits.

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# **Chapter 1** General Introduction

## 1.1 Adaptive maternal effects in oviparous animals: an overview

Maternal effects occur whenever the phenotype of a mother influences the phenotype of her offspring over and above the direct effects of genetic inheritance; a definition that encompasses diverse maternal traits including nest selection, rearing and teaching behaviours and the transfer of material resources. As pervasive sources of phenotypic variation, maternal effects may be targets for selection and may have adaptive roles if they predictably increase maternal fitness in heterogeneous environments (Mousseau & Fox 1998a; Marshall & Uller 2007). Of all maternal traits, perhaps none has received greater attention than egg composition in oviparous animals. In addition to the energetic resources needed to fuel growth, mothers provision their eggs with a cocktail of antioxidants, hormones, immuno-proteins and mRNAs which may provide a toolkit with which to manipulate offspring phenotypes and buffer them against specific environmental stressors (Blount et al. 2000; Groothuis et al. 2005; Boulinier & Staszewski 2008). In many oviparous animals, females also control the developmental environment that their offspring will experience through choice of oviposition sites (Mousseau & Fox 1998b). By providing environmental continuity across generations, maternal oviposition preferences may drive fine-scale adaptation to specific environmental conditions and may therefore be highly adaptive in heterogeneous landscapes (Resetarits 1996).

This thesis examines the adaptive significance of two specific maternal traits in the green turtle (*Chelonia mydas*); namely the provisioning of fat-soluble antioxidants in eggs and the selection of oviposition sites via natal homing. In the following pages I provide relevant background and outline the aims and scope of this thesis.

## 1.2. Egg yolk antioxidants as adaptive maternal effects

### Oxidative stress and the vertebrate antioxidant system

Aerobic metabolism yields significant energetic benefits for living organisms, however it also poses a serious oxidative challenge through the generation of cytotoxic reactive oxygen species (ROS). The majority of these ROS are normal by-products of

mitochondrial activity, formed by 'leakage' of electrons from the respiratory electron transport chain to molecular oxygen. However, they are highly unstable and react quickly with adjacent biomolecules causing indiscriminate damage to lipids, proteins and DNA (Halliwell & Gutteridge 2007). Such damage has been linked with cell death, aging and the etiology of a number of degenerative diseases (Finkel & Holbrook 2000; Christen 2000). To counteract the harmful effects of ROS, animals have evolved a multifaceted antioxidant system consisting of endogenously produced enzymes and metabolites (e.g. catalase, superoxide dismutase, uric acid) in addition to a number of exogenous, diet-derived compounds (e.g. carotenoids, vitamins E and C) (reviewed in Halliwell & Gutteridge 2007; Monaghan et al. 2009). An imbalance between the production of ROS and the capacity of the antioxidant system to neutralise them is described as 'oxidative stress' and can result in widespread cellular damage (Monaghan et al. 2009).

**Oxidative stress in the vertebrate embryo**

Oxidative stress is particularly likely under conditions of rapid metabolism or reduced antioxidant status (Monaghan et al. 2009), which may put developing embryos with high rates of cell division and potentially immature endogenous antioxidant defences particularly at risk (Blount et al. 2000). Studies using *in vitro* embryos have shown that oxidative stress induced by ROS can impair embryonic growth, reduce viability and increase the risk of malformations (Umaoka et al 1992; Burton et al. 2003). Thus, maternal strategies that buffer their offspring from oxidative stress could be highly adaptive. In all oviparous vertebrates, females transfer significant quantities of diet-derived carotenoids and vitamin E into their eggs which may serve to mitigate oxidative stress during development and early life (Blount et al. 2000; Surai 2002; McGraw et al. 2005). Vitamin E and carotenoids are only synthesised *de novo* in plants and other photosynthetic organisms where they function to protect the photosynthetic apparatus from photo-oxidative damage (Demmig-Adams & Adams 1996; Munné-Bosch 2005). However they are also thought to have important functions as fat-soluble antioxidants in animals, such as quenching free radicals and terminating lipid peroxidation chain reactions initiated by ROS that can damage lipid membranes (Sies & Stahl 1995; Traber & Atkinson 2007). These functions may be of particular relevance in the egg yolk and embryos of oviparous animals which typically contain high concentrations of polyunsaturated fatty acids that are especially susceptible to lipid peroxidation (Surai et al 1999). Yolk-derived carotenoids and vitamin E are progressively transferred to the

embryo during the later stages of development and are thought to contribute to the antioxidant system of the neonate during the sudden exposure to atmospheric oxygen tensions and the switch from chorioallantoic to pulmonary respiration which accompany hatching (Gaal et al. 1995; Blount et al. 2000; Surai 2002).

To date, studies of maternal effects mediated by antioxidant deposition in eggs have tended to focus on carotenoids with much less attention directed to vitamin E. I therefore first review evidence for maternal effects related to carotenoid deposition in eggs and then discuss the potential for vitamin E provisioning to influence offspring phenotypes.

**Maternal effects of yolk carotenoids**

Carotenoids were first identified as the source of the yellow pigmentation of egg yolk a century ago (Willstätter & Escher 1911), but it is only recently that the deposition of carotenoids in eggs has attracted the attention of evolutionary ecologists as a potentially important source of maternal effects (Blount et al. 2000). In the past decade a large number of studies have examined the effects of yolk carotenoid concentrations on offspring growth, survival and immune function in birds and fish, but have yielded inconsistent and often contradictory results (summarised in **Table 1.1**). Other studies have reported taxon-specific effects on nestling begging intensity and plumage colouration (Isaksson et al. 2006; Helfenstein et al. 2008; Biard et al. 2009) which, while interesting, cannot account for the ubiquitous distribution of carotenoids in the eggs of birds, fish, reptiles and many insects (Goodwin 1950). The beneficial effects of yolk carotenoids on offspring body size and immune function have proven most repeatable (but still highly equivocal; **Table 1.1**), although it is unclear whether such effects relate to the antioxidant protection afforded to embryos or some other non-antioxidant role (Haq et al. 1996; Biard et al. 2005; Saino et al. 2008). Indeed, while studies have repeatedly shown that increased yolk carotenoid concentrations can reduce the susceptibility of yolk and embryonic tissues to lipid peroxidation *in vitro* (Surai & Speake 1998; Blount et al. 2002a, b; McGraw et al. 2005), it is unknown whether this protective effect for offspring is replicated *in vivo*. This is an important omission given that recent work in birds and reptiles has cast doubts on the capacity of carotenoids to suppress oxidative damage under physiological conditions (Constantini & Møller 2008; Isaksson & Andersson 2008; Olsson et al. 2008), suggesting that the antioxidant role of maternally-derived carotenoids in eggs may need to be revaluated.

**Table 1.1.** A summary of maternal effects mediated by carotenoid provisioning in eggs reported for oviparous animals. Ticks denote a significant main effect ($p \leq 0.05$), ticks in parentheses denote significance only in interaction with other variables, and crosses denote no effect found.

| Species | Hatching success | Growth | | Cell mediated immunity* | Survival† | Ref |
|---|---|---|---|---|---|---|
| | | Size | Mass | | | |
| **Birds** | | | | | | |
| *F*alco *tinnunculus* | × | × | × | × | × | 1 |
| *Parus major* | × | × | × | × | × | 2 |
| | - | - | - | (✓) | - | 3 |
| | - | × | (✓) | (✓) | - | 4 |
| | × | × | (✓) | - | - | 5 |
| *Taenopygia guttata* | ✓ | - | - | - | ✓ | 6 |
| *Parus caeruleus* | × | ✓ | × | - | × | 7 |
| | - | × | × | (✓) | - | 8 |
| *Serinus canaria* | × | - | ✓ | × | × | 9 |
| *Hirundo rustica* | × | × | × | ✓ | - | 10 |
| *Notiomystis cincta* | × | ✓ | × | × | × | 11 |
| | × | (✓) | (✓) | - | × | 12 |
| *Larus michahellis* | - | ✓ | × | × | - | 13 |
| *Perdix perdix* | - | - | × | × | - | 14 |
| *Gallus gallus domesticus* | - | - | - | (✓) | - | 15 |
| **Fish** | | | | | | |
| *Gobiusculus flavescens* | × | × | - | - | × | 16 |
| *Poecilia reticulata* | - | × | × | - | - | 17 |

*Assessed using a cutaneous hypersensitivity response induced by phytohemaglutinin (PHA).
†Post-hatching survival to fledging (birds) or over time (fish)

Refs: 1) de Neve et al 2008; 2) Remes et al 2007; 3) Berthouly et al 2007; 4) Berthouly et al. 2008a; 5) Berthouly et al 2008b; 6) McGraw et al. 2005; 7) Biard et al. 2005; 8) Biard et al. 2007; 9) Tanvez et al 2009; 10) Saino et al. 2003; 11) Ewen et al 2008; 12) Ewen et al. 2009; 13) Saino et al. 2008; 14) Cucco et al. 2006; 15) Koutsos et al. 2007; 16) Svensson et al. 2006; 17) Grether et al. 2008; N.B. The table summarises studies with an ecological or evolutionary focus (i.e. maternal effects) and does not attempt to review the sizeable literature from the poultry and aquaculture industries.

## Maternal effects of yolk vitamin E

As argued in a recent review (Catoni et al. 2008), the focus on carotenoids in evolutionary ecology may have detracted attention from other, more potent diet-derived antioxidants. For example, while lacking the visual appeal of carotenoids, vitamin E is typically deposited at far higher concentrations in the eggs of birds and reptiles (Surai 2002; Thompson & Speake 2004) and has an undisputed role as an antioxidant in

animals (Traber & Atkinson 2007; Halliwell & Gutteridge 2007). Moreover, vitamin E has an established requirement in reproduction, having been first discovered because deficiency in the maternal diet invariably results in early embryonic death (Evans & Bishop 1922): a phenomenon which has since been attributed to the antioxidant function of vitamin E in protecting embryos from oxidative stress (Draper et al. 1964). Maternal vitamin E deficiency is also frequently associated with reduced hatchability of eggs in domestic poultry (reviewed in Surai 2002). However, despite these credentials, vitamin E deposition in eggs has been little explored as a source of maternal effects in wild animals.

Several studies in domestic chickens have shown that higher vitamin E concentrations in eggs can reduce the susceptibility of yolk and embryonic tissues to lipid peroxidation *in vitro* (Chen et al. 1998; Surai et al. 1999), although as with carotenoids it is not known whether this protective effect is replicated *in vivo*. Only a single study has investigated the fitness consequences of variable vitamin E provisioning in eggs in the wild, and found that higher yolk concentrations were associated with increased hatching success of barn swallow (*Hirundo rustica*) eggs in radioactively damaged habitats (Møller et al. 2008). There have also been several experimental tests of the effects of dietary vitamin E on early development in birds and reptiles which have provided mixed results. Supplemental vitamin E has been shown to enhance the growth of nestling birds which was attributed to the extra antioxidant protection afforded (de Ayala et al. 2006; Hall et al. 2010); however, a study using subcutaneous implants to deliver a sustained dose of vitamin E in young lizards found no effects on survival rates over an extended period (Healey & Olsson 2009). Clearly there is much still to be learned about the functional consequences and adaptive role of maternal vitamin E deposition in eggs.

**Is antioxidant deposition in eggs adaptive?**

Despite considerable interest in the ultimate consequences of maternal antioxidant deposition in eggs for offspring, it is essentially unknown whether mothers exert any active control over this process and why levels of provisioning vary naturally among individual females. Since all animals must ultimately obtain carotenoids and vitamin E from the diet, it is often suggested that they may be limiting resources for egg production (Royle et al. 2001; Blount et al. 2004; Biard et al. 2005). However, while it is well established that supplementing the maternal diet with carotenoids and vitamin E increases the deposition of these compounds in eggs (Blount et al. 2002a; Grobas et al.

2002; Royle et al. 2003), there is actually very little evidence that individuals vary in their ability to acquire dietary antioxidants under natural conditions (Hudon 1994; Catoni et al. 2008). On the contrary, studies of carotenoid acquisition for use in brightly coloured plumage displays in birds suggest that diet is a negligible source of variation among individuals (McGraw & Hill 2001; Hadfield & Owens 2006).

Evidence that females adaptively adjust the antioxidant content of their eggs to modify offspring phenotypes is similarly lacking. Most species of birds show pronounced reductions in the quantities of vitamin E and carotenoid deposited in eggs across the laying sequence, which is hypothesised to be an adaptive strategy to handicap lower-quality, late hatching chicks (Royle et al. 1999; Hõrak et al. 2002), or to complement opposing within-clutch patterns in steroid hormone deposition (Royle et al. 2001). However, such trends could equally arise passively through the depletion of maternal antioxidant reserves (Badyaev et al. 2006; Groothuis et al. 2006). Since little is known about patterns of within-female antioxidant provisioning to eggs in other taxa it is difficult to differentiate between these hypotheses. Several studies of wild birds which experimentally increased carotenoid levels in eggs found beneficial effects only in certain environmental contexts (e.g. high levels of parasitism or sibling competition; Berthouly et al. 2008a; Ewen et al. 2009; Hall et al. 2010), although whether females naturally respond to such cues by adjusting carotenoid investment in eggs is not known. A single study in gulls showed that females exposed to more intraspecific aggressive contacts deposited more carotenoids in eggs, but this result could not be disentangled from confounding effects on maternal physiological state (Verboven et al. 2005). More work is therefore required to determine which aspects of the offspring developmental environment affect exposure to oxidative stress, how this influences the requirement for (and effects of) maternally-derived antioxidants, and whether females respond to such cues in order to optimise the antioxidant content of their eggs.

## 1.3. Nest site selection and natal homing as adaptive maternal effects

Where and when a female lays her eggs is often the single most important determinant of reproductive success for oviparous animals (Mousseau & Fox 1998b), particularly those species lacking parental care where mothers cannot compensate for poor quality oviposition environments through post-laying behaviour. Oviposition site choice can

affect the abiotic environment experienced by offspring, the availability of food, and the level of predation and competition for resources they encounter (van Buskirk & McCollum 1999; Rieger et al. 2004; Kamel & Mrosovsky 2005). Since offspring will generally be adapted to a specific set of environmental conditions, maternal oviposition behaviours that match offspring phenotypes to the local environment may be highly adaptive. This is supported by the (perhaps unsurprising) finding that when confronted with a choice of potential ovipoisition sites, female insects, reptiles and amphibians often select the site in which offspring fitness is maximised (Craig et al. 1989; Rieger et al. 2004; Brown & Shine 2004; Dvořák & Gvoždík 2009). The coupling of offspring performance and maternal oviposition preferences has been modelled as a coadaptive process, in which the genes for maternal oviposition behaviours evolve in concert with offspring genes that are adapted to the maternally-provided environment (Wade 1998; Wolf 2000). Thus, maternal oviposition behaviour may play a pivotal role in life history evolution and local adaptation of offspring by maintaining environmental continuity across generations (Resetarits 1996).

As an alternative to actively discriminating among oviposition sites, a stable selective environment could also be achieved if females return to lay their eggs at the particular site where they themselves were born ('natal homing'). In this way the offspring developmental environment is likely to resemble that of the mother, facilitating adaptation to specific environmental regimes (Resetarits 1996). Natal homing has been well documented in certain groups of species such as the salmonid fish and marine turtles (Quinn & Dittman 1990; Bowen & Karl 2007), but is a relatively widespread phenomenon in diverse animal taxa (marine fish: Thorrold et al. 2001; salamanders: Gamble et al. 2007; anurans: Berven & Grudzien 1990; snakes: Brown & Shine 2007; and birds: Wheelwright & Mauck 1998). However, despite its prevalence among oviparous animals, the possibility that parental homing behaviour facilitates the adaptation of offspring traits to the natal habitat has only previously been explored in anadromous fish (reviews in Quinn & Dittman 1990; Taylor 1991; Dittman & Quinn 1996). In these species, larvae and juvenile fish exhibit a multitude of local adaptations to their natal environments and have increased viability in comparison to fish born at other sites (Taylor 1991). One of the principle aims of this thesis is to establish whether maternal homing behaviour in marine turtles similarly results in locally adapted offspring phenotypes, which may help to explain the adaptive basis of homing behaviour in these species.

## 1.4 Maternal effects in the green turtle

The green turtle (*Chelonia mydas*) is one of seven species of marine turtles and is circumglobally distributed in tropical and subtropical oceans. Females breed at intervals of 2-4 years, typically returning to the particular nesting beach or colony from which they themselves were hatched (Miller 1997). As with other marine turtles, green turtles do not exhibit post-laying parental care meaning maternal effects are largely limited to investment in eggs and the selection of nest sites. Females are highly fecund, producing as many as 600 eggs within a single breeding season divided across 2-5 large clutches (van Buskirk & Crowder 1994), which is presumably a life-history adaptation to the low survival rates of hatchlings, estimated to be between 0.1 - 0.01% (summarised in Frazer 1986). However, besides a well established correlation between egg size and hatchling size (e.g. van Buskirk & Crowder 1994), very little is known about how other aspects of egg composition influence offspring phenotypes in marine turtles. Maternally-derived resources may be of particular importance for hatchling turtles, which soon after hatching embark on a phase of high activity in order to dig free from the nest chamber in which they are buried and swim offshore to reach open ocean habitats. During this time hatchlings do not feed and metabolic rate may be 3 to 4 times basal levels (Wallace et al. 2008), which may pose a high risk of oxidative stress and create a requirement for yolk-derived antioxidants.

Female sea turtles bury their eggs in nests excavated in sandy, colonial nesting beaches where embryonic development is profoundly affected by the abiotic properties of the beach (e.g. gas conductance, moisture potential) and local climatic conditions (Ackerman 1997). As with all ectotherms, incubation temperature dictates many aspects of marine turtle development including growth rates, embryonic viability, hatchling morphology and locomotor performance and sex (reviews in Birchard 2004; Booth 2004). In sea turtles sex is also temperature dependent with increasing proportions of females produced at higher incubation temperatures (Godley et al. 2002). Maternal choice of nest sites may therefore have a significant impact on offspring phenotypes. Nest selection in marine turtles can be viewed as a hierarchical phenomenon in which females first return to their natal colony and then at some point homing behaviour gives way to interactive nest site choice. It is unclear which specific cues females use to select nest sites once emerged onto the nesting beach, and whilst some studies have suggested that nest selection is an individually consistent trait (e.g. Kamel & Mrosovsky 2005),

others suggest that it may be a largely random process (Bjorndal & Bolten 1992). However, given that environmental conditions within a particular nesting beach are likely to be less variable than among different nesting beaches (e.g. Hays et al. 1995), the homing component of nest selection may often be the single most important determinant of the offspring developmental environment.

All research on wild turtles in this thesis was conducted at Ascension Island, an isolated volcanic peak in the South Atlantic Ocean (**Figure 1.1**) which hosts one of the largest Atlantic breeding populations of green turtles. Turtles nesting at this site migrate over 2000 km from feeding pastures along the coast of Brazil (Hays et al. 2002a) and are genetically distinct from other Atlantic populations (Meylan et al. 1990), indicating that most (if not all) breeding individuals are born at Ascension Is. themselves.

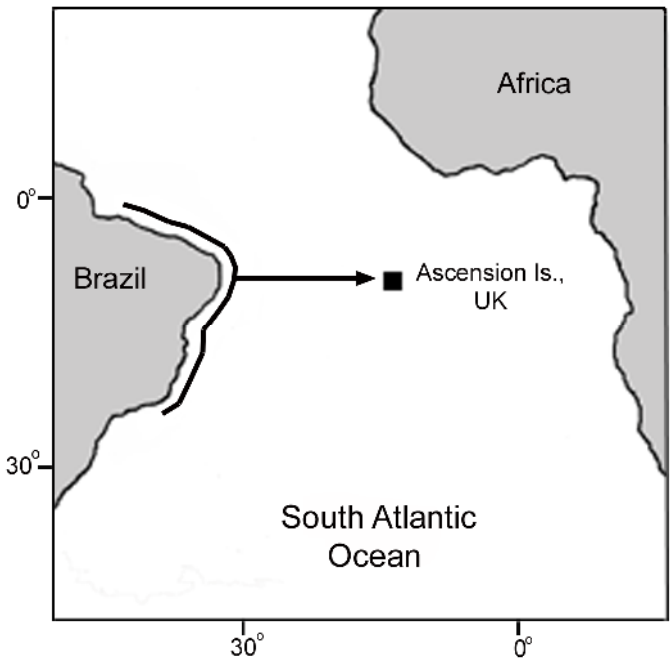



**Figure 1.1.** Location of Ascension Island and approximate migration routes of green turtles that nest there (bold arrows; Carr 1967; Hays et al. 2002a).

## 1.5 Aims and structure of the thesis

This thesis examines the adaptive significance of maternal antioxidant deposition in eggs, and nest site selection via natal homing in the green turtle. An additional chapter is also presented examining the effects of ambient temperature on egg production rates and maternal behaviour in this species.

The thesis begins with a major section examining the natural sources of variation in the fat-soluble antioxidant content of green turtle eggs and the functional consequences of this variation for offspring (**Chapters 2-5**). First, **Chapter 2** addresses some methodological issues associated with extracting carotenoids from lipid rich egg yolk and describes a novel approach for isolating carotenoids from yolk lipids which greatly improves chromatographic separations. **Chapters 3 and 4** then explore some of the proximate mechanisms underlying intraspecific variation in the antioxidant content of green turtle eggs. Specifically, in **Chapter 3** the hypothesis that maternal dietary access to antioxidants underpins variable antioxidant deposition in eggs is tested using a comparison of wild and captive green turtles with contrasting dietary regimes (natural diet *vs*. uniform diet). The chapter further examines how antioxidant levels vary within and among successive clutches laid by individual females over the course of the nesting season and discusses the possible adaptive significance of such trends. **Chapter 4** extends the analysis of mechanisms by relating the vitamin E and carotenoid content of green turtle eggs to maternal plasma concentrations, and investigates whether variable antioxidant levels in eggs are quantitatively transferred to hatchlings. **Chapter 5** then turns to the functional consequences of antioxidant provisioning to eggs for offspring. A correlative study is presented that examines how yolk antioxidant levels and the maternally-provided nest environment combine to determine oxidative stress levels in hatchling turtles, and investigates whether female turtles tailor the antioxidant content of their eggs to a specific context.

Changing focus, **Chapter 6** takes an experimental approach to explore the adaptive advantages of natal homing behaviour in female green turtles. Using a common-garden rearing experiment, I test the hypothesis that maternal homing behaviour facilitates local adaptation offspring to the thermal environment of the natal nesting beach. **Chapter 7** deviates somewhat from the theme of maternal effects, but continues the narrative of temperature as an important influence on marine turtle life-history, by investigating how temperature affects the rate at which females produce eggs. In particular, the chapter tests the temperature-dependence of egg production against the predictions of the Metabolic Theory of Ecology (Brown et al. 2004), and discusses how the relationship between temperature, metabolic rate and egg formation may have shaped maternal reproductive behaviour. Finally, **Chapter 8** presents a synthesis of the results and assesses the adaptive significance of maternal antioxidant deposition in eggs and nest selection via natal homing.

# Chapter 2 A novel approach for the extraction of carotenoids from egg yolk.

## 2.1 Abstract

In recent years, there has been growing interest in the role of maternally-derived carotenoids in the eggs of oviparous animals, which has required accurate methods for the quantitative extraction of carotenoids from egg yolk. Nonetheless, current liquid-liquid extraction (LLE) approaches have limitations which may render them unsuitable for some applications. In particular, large amounts of yolk lipid are also recovered during LLE which can interfere with chromatographic analysis of carotenoids. Here I outline a novel approach for isolating carotenoids from yolk lipids using solid phase extraction (SPE) on diol-silica cartridges. The procedure is simple, rapid and significantly improves chromatographic separations of yolk carotenoids for species where the ratio of carotenoids to lipids in eggs is low. Recoveries of common yolk xanthophyll carotenoids (polar carotenoids) using SPE were high and comparable to recoveries obtained using LLE. Carotenes (non-polar carotenoids) were not recovered by SPE, however it was nonetheless possible to quantify carotene levels in egg yolk by analysis of the eluted lipid fraction. I suggest that SPE may be a useful sample pre-treatment step for the analysis of yolk carotenoids in some species of birds and many reptiles where carotenoids are present at low concentrations.

## 2.2 Introduction

Carotenoids are a diverse family of naturally occurring, fat soluble pigments responsible for the familiar yellow-red colouration of egg yolk in many species of birds, reptiles and fish (Blount et al. 2000). The carotenoid lutein was first identified in eggs of the domestic chicken a century ago (Willstätter & Escher 1911) and manipulation of the carotenoid content of egg yolk has since become a mainstay of the poultry industry in order to satisfy consumer tastes for brightly coloured produce (Palmer & Kempster 1919; Fletcher 1992; Hencken 1992; Leeson & Catson 2004). More recently, the provisioning of carotenoids in eggs has also attracted the attention of evolutionary ecologists due to their potential to mediate maternal effects in oviparous animals (e.g. Blount et al. 2002a; Biard et al. 2005; McGraw et al. 2005). Maternally-derived carotenoids in eggs have been linked with a range of beneficial effects for offspring, including antioxidant and immunomodulatory properties, which may impact on the health of the developing embryo and neonate (Surai 2002; Saino et al. 2003; **Table 1.1**). Robust methods for the quantitative extraction of carotenoids from egg yolk are therefore fundamental to a wide range of commercial and academic interests; however current approaches suffer from limitations which in some cases make them incompatible with the modern chromatographic techniques used in carotenoid analysis.

High performance liquid chromatography (HPLC) has become the standard tool for the separation and quantification of carotenoids, but requires that the carotenoids are first isolated from the sample matrix. The extraction of carotenoids from egg yolk has traditionally involved a liquid-liquid extraction (LLE) procedure, in which the carotenoids are first solubilised in a polar solvent (e.g. ethanol, acetone) and then partitioned into a non-polar solvent system such as petroleum spirit, hexane or tetrahydrofuran (Surai & Speake 1998; Bortolotti et al. 2003; Isaakson et al. 2006). This approach effectively separates carotenoids from yolk proteins and other hydrophilic components; however the majority of the yolk lipid (largely triacyglycerol) is also transferred to the non-polar phase and contaminates the resulting extract. Such lipids are generally insoluble in the polar eluents used in reverse phase HPLC meaning that LLE extracts must be analysed dissolved in less polar solvent mixtures (Surai & Speake 1998). Whilst this is often effective at low levels of lipid contamination, excess lipid may impair chromatographic separations and may precipitate on contact with the eluent thus reducing the lifespan of analytical columns. Additional sample pre-treatment to

isolate carotenoids from yolk lipids may therefore be advantageous for some applications.

Separating carotenoids from fatty tissues is not a new problem and has generally been accomplished using alkaline saponification to hydrolyse any lipids in the sample (e.g. Negro et al. 2001a; El Sohemy et al 2006). However, carotenoids are highly labile compounds and the oxidising conditions employed in saponification may be associated with significant carotenoid degradation or isomerisation (e.g. lutein → cis-lutein; Schiedt & Liaaen Jensen 1995; Lietz & Henry 1997; Negro et al. 2001a; McGraw & Toomey 2010). Furthermore, the need to thoroughly remove traces of alkali from saponification extracts prior to analysis makes the process time consuming and not conducive with high sample throughput (Schiedt & Liaaen-Jensen 1995). In this chapter I describe a novel approach for isolating yolk carotenoids from contaminating lipids using solid phase extraction (SPE) on diol-silica cartridges. SPE has been successfully used for carotenoid analysis in various foodstuffs (e.g. Fisher & Rouseff 1986; Mateos & Garcia Mesa 2006; Shen et al. 2009), but has never been applied to the analysis of egg yolk carotenoids. The described method is simple, rapid and yields comparable recoveries of many common yolk carotenoids to the traditional LLE approach. To demonstrate the potential benefits of SPE over LLE for certain ecological applications, HPLC separations of yolk carotenoids from the eggs of several species of birds and reptiles are presented following extraction by each method.

## 2.3 Materials & Methods

### 2.3.1 Preparation of standards

The quantitative recovery of carotenoids by different extraction methods was assessed using standard solutions of lutein, β-cryptoxanthin, canthaxanthin and β-carotene (Sigma Chemical Co., St Louis, MO; CaroteNature, Lupsingen, Switzerland), as these carotenoids are commonly found in the eggs of avian and reptilian species (Surai 2002; Blount et al. 2002b; Dierenfeld et al. 2002; Cassey et al. 2005). Standards were prepared in hexane or petroleum spirit and concentrations verified spectrophotometrically using specific extinction coeffecients and absorbance maxima suggested by Britton et al. (2004). Standards were then evaporated to dryness, reconstituted in methanol and combined to produce a working standard containing 1 µg $ml^{-1}$ of each compound.

### 2.3.2 Collection of egg samples

In order to cover a wide range of possible applications, eggs for carotenoid analysis were obtained from a variety of common avian study species (including wild and captive populations) and a single species of reptile. The species chosen were: great tits (*Parus major*), domestic chickens (*Gallus gallus domesticus*), zebra finches (*Taeniopygia guttata*) and green turtles (*Chelonia mydas*). Great tit eggs ($N$ = 3) were collected from a box breeding population in Devichoys Wood, Cornwall, UK (5º7′W, 50º12′N) in April 2009. Zebra finch eggs ($N$ = 3) were collected from an aviary population maintained at the University of Exeter and fed an *ad libitum* seed-based diet. Eggs from free range domestic chickens (*Gallus gallus domesticus*) were sourced from a local farm in Cornwall, UK ($N$ = 3). Green turtle eggs ($N$ = 3) were collected from females nesting at Ascension Island, South Atlantic Ocean (14º20'W, 7º55'S) in April 2007. With the exception of the domestic chicken where laying females could not be identified, all eggs were collected from different individuals. Yolk and albumen fractions were carefully separated using a domestic egg separator (turtle and chicken) or moistened filter paper (finches and tits), and aliquots of the homogenised yolk were stored at - 80 ºC prior to analysis.

### 2.3.3 Liquid-liquid extraction of yolk carotenoids

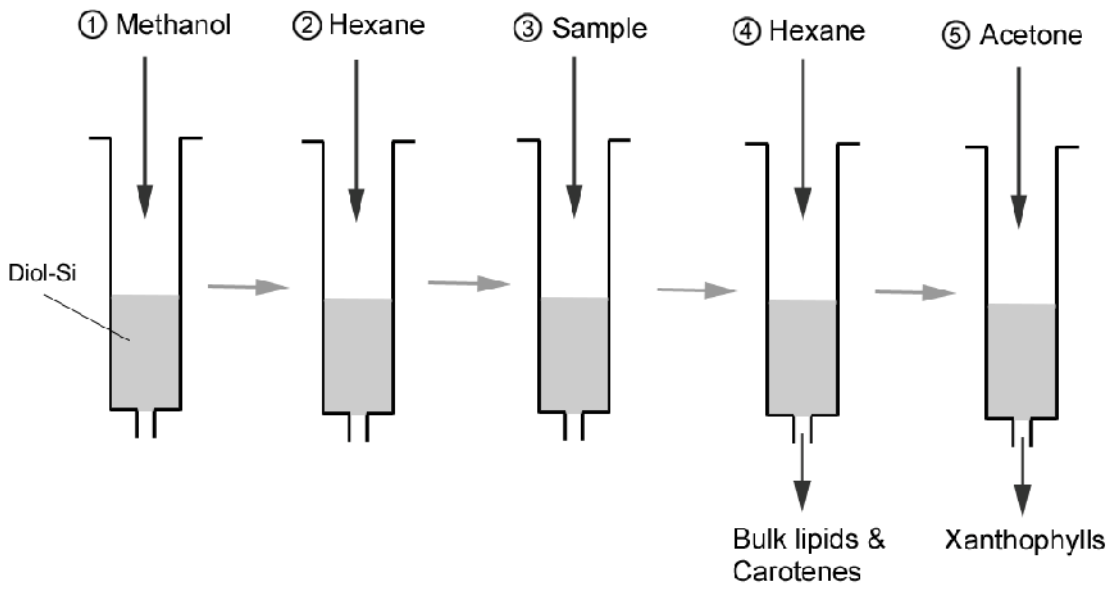
Liquid-liquid extraction (LLE) was carried out using previously described methods for the analysis of yolk carotenoids (Surai & Speake 1998; Blount et al. 2002b). A pre-weighed sample of frozen egg yolk (chicken/turtle: 200-300 mg; tits/finches: 50-100 mg) was first vortex mixed with 1 ml 5 % sodium chloride (w/v in distilled water) and homogenised in 1ml ethanol. For recovery tests, 0.5 ml of the combined carotenoid standard (§ 2.3.1) replaced 0.5 ml of the ethanol in this initial mixture ($N$ = 3 assays). The carotenoids were then extracted by further homogenisation of the sample with 2 ml hexane followed by centrifugation for 5 mins at 10,000 × g to separate the phases. The organic (upper) phase containing the carotenoids and yolk lipids was recovered and the yolk residue re-extracted with an additional 2 ml hexane. The pooled organic phases from both extractions were then dried on a rotary evaporator and redissolved in 0.5 ml methanol / dichloromethane (1:1 v/v) ready for HPLC analysis (§ 2.3.5).

### 2.3.4 Solid phase extraction of yolk carotenoids

As an additional sample pre-treatment step, carotenoids extracted using LLE were further isolated from contaminating yolk lipids by solid phase extraction (SPE) on

Discovery® diol-bonded silica cartridges (500mg/3ml; Supelco, Bellefonte, PA; **Figure 2.1**). Cartridges were prepared by preconditioning with consecutive washes of 3 ml methanol followed by 3 ml hexane and the sorbent bed was wrapped with aluminium foil to protect carotenoids bound to it from photooxidation. Dried carotenoid/lipid extracts obtained by LLE of each yolk sample or standard (as described in § 2.3.5) were then redissolved in 200 µl hexane and applied to the column. Contaminating lipids were eluted with the addition of a further 3 ml hexane forced through the column under positive pressure. Finally, carotenoid pigments bound to the column were recovered in 3 ml acetone applied in consecutive 1 ml aliquots. Both hexane and acetone fractions were collected and dried on a rotary evaporator. Acetone fractions containing the carotenoids were reconstituted in 0.5 ml of 85:15 acetonitrile/methanol (v/v) ready for HPLC. In order to assess carotenoid losses from the column, the hexane fractions containing the yolk lipids were also redissolved in 0.5 ml methanol / dichloromethane (1:1 v/v) and analysed by HPLC (see **Figure 2.1**).

**Figure 2.1.** Outline of the solid phase extraction procedure for isolating carotenoids from yolk lipids. The column is preconditioned with methanol and hexane (1-2), the sample is applied dissolved in hexane (3) and the analytes are eluted firstly with hexane (4), and then acetone (5). Carotenes (unoxygenated carotenoids) are eluted in the hexane fraction along with lipids while xanthophylls (oxygenated carotenoids) are recovered in the acetone fraction.



### 2.3.5 High performance liquid chromatography

Carotenoids were analysed by HPLC using a 250 × 4.6 mm Spherisorb S5ODS2 5 µm C18 reverse phase HPLC column (Waters Ltd., Dublin) and mobile phases of 85:15 acetonitrile/methanol (v/v) and 70:20:10 acetonitrile/dichloromethane/methanol (v/v/v) in gradient elution (as described by Granado et al 1998). A 50 µl injection volume was used for carotenoid extracts from bird eggs and an 80 µl volume for turtle eggs. Detection was by absorbance at 445 nm. Peaks were identified by comparison to the retention times of carotenoid standards and concentrations of each compound in the extract determined from peak areas against a calibration curve prepared using serial dilutions of the standard. Carotenoids which could not be identified using the available standards were calibrated against the standard curve for lutein.

## 2.4 Results and Discussion

The extraction of carotenoids from egg yolk has traditionally been accomplished using liquid-liquid extraction (LLE) which also recovers the majority of the yolk lipids. This study tested a novel approach for isolating yolk carotenoids from contaminating lipids using solid phase extraction (SPE) on diol-silica columns. The benefits of removing yolk lipids prior to HPLC analysis of carotenoids are self evident from a comparison of the chromatograms obtained for LLE and SPE extracts of egg yolk from several species of birds and reptile (**Figure 2.2**). In all cases, SPE pre-treatment significantly improved peak resolution allowing for more reliable identification and quantification of individual yolk carotenoids. Interestingly, contaminating lipids only interfered with HPLC separations of polar carotenoids (xanthophylls) with retention times < 12 minutes, and not the less polar carotenes with longer retention times. This is well illustrated by chromatograms obtained for LLE extracts of great tit eggs which had a complex profile of carotenes eluting between 14 - 19 minutes (**Figure 2.2D**).

The diol-silica sorbent chosen for SPE strongly retained polar xanthophyll carotenoids (lutein, canthaxanthin, and β-cryptoxanthin) and yielded similar recoveries of external standards of these compounds compared to LLE (**Table 2.1**). However the sorbent had no affinity for non-polar β-carotene, the vast majority of which was recovered in the lipid-containing hexane fraction from SPE (**Figure 2.1** and **Table 2.1**). Carotenes are

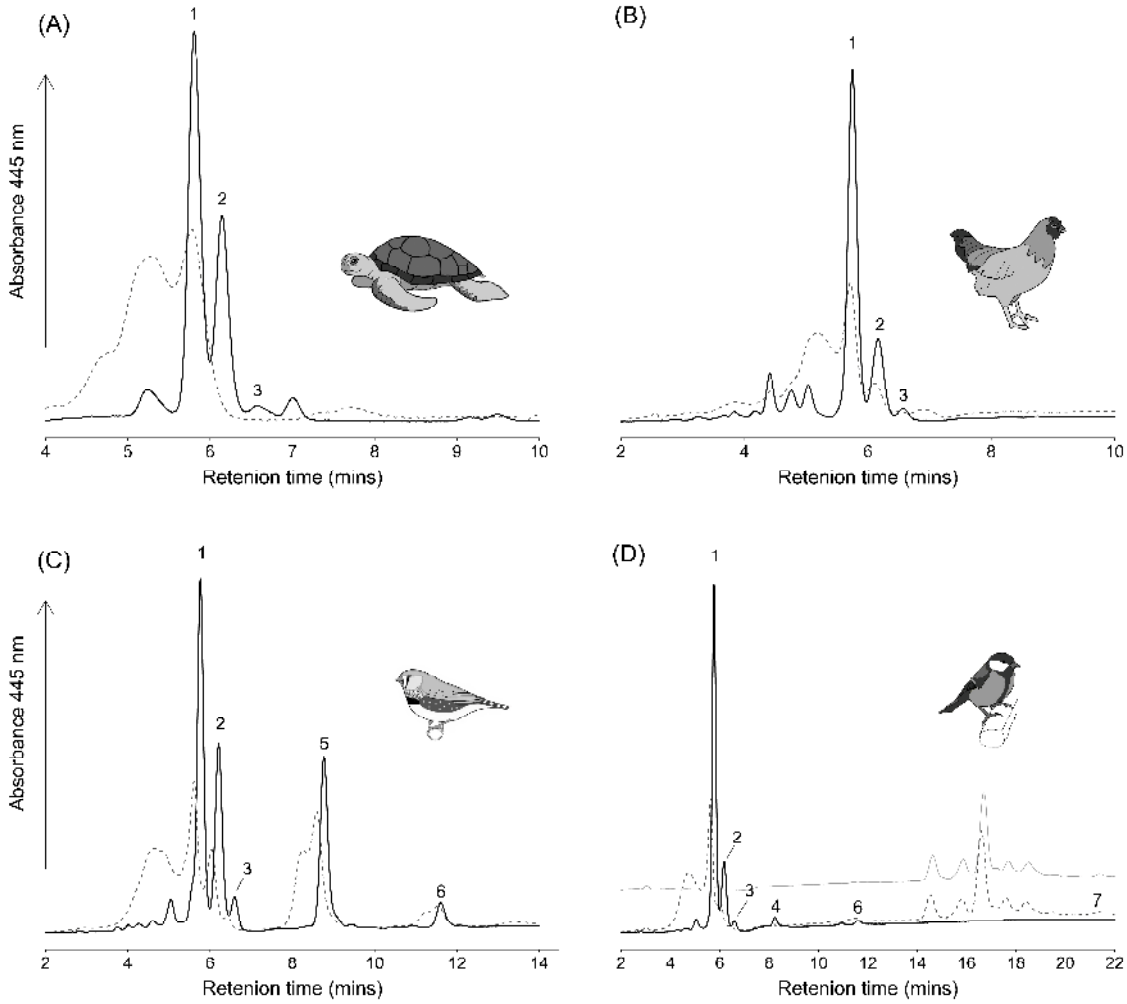



**Figure 2.2.** HPLC chromatograms of egg yolk carotenoids from various species extracted using LLE (broken lines) or SPE (solid lines): (A) green turtle, *Chelonia mydas*; (B) domestic fowl, *Gallus gallus domesticus*; (C) zebra finch, *Taeniopygia guttata*; (D) great tits, *Parus major*. The faded line in panel (D) shows a chromatogram for the SPE hexane fraction containing carotenes and yolk lipids (step 4 in **Figure 2.1**) offset to allow comparison. Numbered peaks correspond to 1) lutein; 2) zeaxanthin; 3) *cis*-zeaxanthin; 4) dehydrolutein; 5) anhydrolutein; 6) β-cryptoxanthin; 7) β-carotene; other compounds could not be identified with available standards.

typically present at low levels in avian eggs in comparison to xanthophylls (Surai 2002), although there are some species where β-carotene accounts for a significant proportion of the total yolk carotenoids (e.g. Surai et al. 2001; Blount et al. 2002b). Nonetheless, since contaminating lipids do not appear to interfere with HPLC separations of carotenes, if necessary the SPE hexane fraction can be analysed separately to identify and quantify carotenes eluted from the cartridge (**Figure 2.2D**). Using this approach, carotenes were only identified in great tit eggs and were not present at detectable levels in the eggs of green turtles, domestic chickens or zebra finches (**Table 2.2**).

**Table 2.1.** Percentage recoveries for external standards of several common yolk carotenoids following either liquid-liquid extraction (LLE) or solid phase extraction (SPE). Recoveries were calculated by comparison of HPLC chromatogram peak areas for each carotenoid relative to the raw standard (retention times are included for reference). Values are mean ± SE of three assays.

| Carotenoid | Retention time (mins) | Recovery (%) | | |
|---|---|---|---|---|
| | | LLE | SPE (acetone fraction) | SPE (hexane fraction) |
| lutein | 5.8 | 94.0 ± 1.7 | 92.8 ± 1.0 | 0.2 ± 0.1 |
| canthaxanthin | 7.3 | 97.4 ± 1.8 | 96.9 ± 1.6 | 0.0 |
| β -cryptoxanthin | 11.6 | 91.8 ± 0.8 | 90.4 ± 1.2 | 0.3 ± 0.1 |
| β-carotene | 21.4 | 89.0 ± 0.5 | 0.0 | 89.8 ± 0.7 |

Despite the clear advantages of removing contaminating lipids from yolk extracts, such a step was not always necessary to achieve adequate separations of yolk carotenoids by HPLC. For example, for great tit and zebra finch eggs which contained high concentrations of carotenoids (**Table 2.2**), acceptable chromatography was also achieved by reducing sample injection volumes of LLE extracts from 50 µl to 10 µl and thus limiting the amount of interfering lipid loaded onto the HPLC column (**Figure 2.3**).

**Table 2.2.** Carotenoid concentrations in the egg yolk of several species of birds and reptiles extracted by SPE. Concentrations are mean ± SE of $N = 3$ eggs per species and are expressed in µg/g of wet yolk. Numbered carotenoids correspond to peaks labelled in **Figure 2.2**.

| Carotenoid | Green turtle | Domestic hen | Great tit | Zebra finch |
|---|---|---|---|---|
| (1) lutein | 2.41 ± 0.3 | 5.49 ± 0.5 | 19.2 ± 1.9 | 34.7 ± 1.6 |
| (2) zeaxanthin | 0.89 ± 0.2 | 1.58 ± 0.1 | 3.20 ± 1.2 | 18.6 ± 3.3 |
| (3) *cis*-zeaxanthin | 0.11 ± 0.04 | 0.28 ± 0.02 | 0.46 ± 0.2 | 2.97 ± 0.3 |
| (4) dehydrolutein | n.d. | n.d. | 0.50 ± 0.2 | n.d. |
| (5) anhydrolutein | n.d. | n.d. | n.d. | 20.8 ± 1.3 |
| (6) β-cryptoxanthin | n.d. | n.d. | 0.31 ± 0.1 | 2.72 ± 0.1 |
| (7) β-carotene | n.d. | n.d. | 0.15 ± 0.08 | n.d. |
| unidentified | 0.35 ± 0.06 | 1.68 ± 0.6 | 15.2 ± 1.7 | 2.96 ± 0.2 |
| **Total** | **3.76 ± 0.5** | **9.03 ± 0.8** | **38.6 ± 2.5** | **82.7 ± 7.1** |

n.d. = none detected

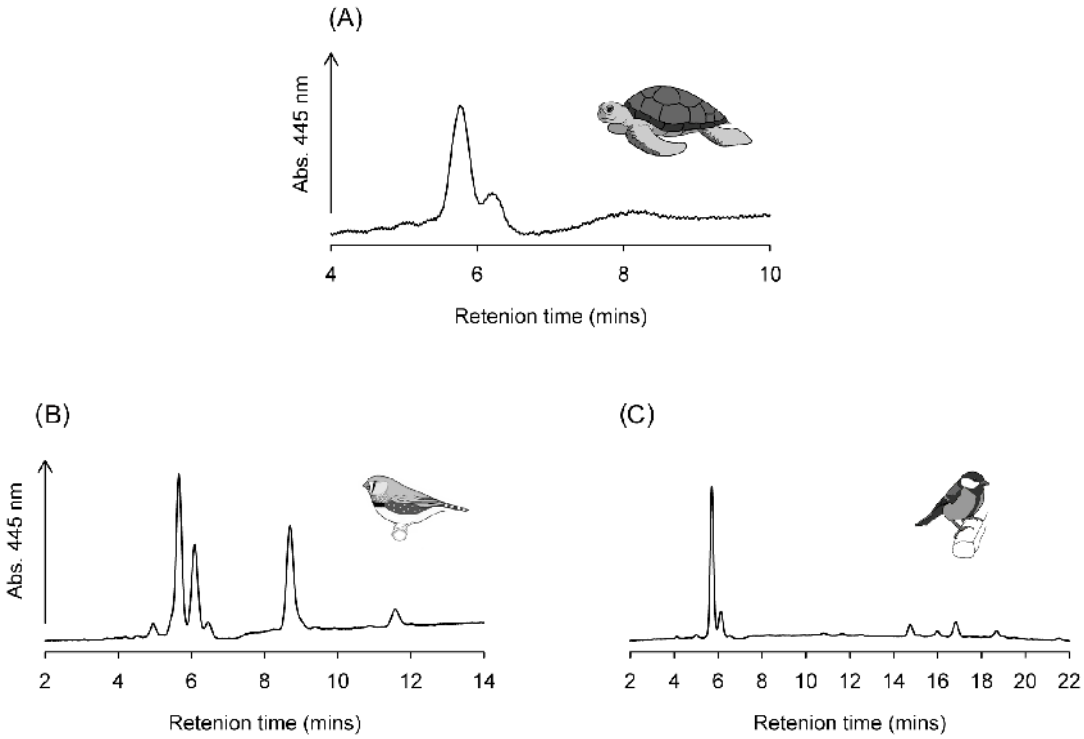



**Figure 2.3** HPLC chromatograms of egg yolk carotenoids from various species obtained following LLE using a low sample injection volume (10 µl).

However, for green turtle eggs in which carotenoid concentrations were an order of magnitude lower than for the passerine species (**Table 2.2**), such low sample volumes contained undetectable levels of many yolk carotenoids and HPLC separations were poor compared to that of SPE extracts (**Figure 2.3**). Even for the passerine species, the ability to discriminate among some of the smaller carotenoid peaks was reduced when using low injection volumes. Thus, SPE pre-treatment is likely to be of particular value for carotenoid analysis in species where yolk carotenoids are present at low levels or when the concentration of quantitatively more minor compounds is of interest.

The exceptionally low level of carotenoids in green turtle eggs relative to those of the species of birds studied was particularly striking (**Table 2.2**). Interspecific variation in yolk carotenoid levels has been well documented in birds (**Figure 2.4**) - some species having concentrations lower than those of green turtle eggs - which raises interesting questions about the evolutionary and ecological factors that regulate maternal carotenoid provisioning (see Cassey et al. 2005; Biard et al. 2009). By comparison, far less is known about the carotenoid content of reptilian eggs; although as in green turtles, species for which measurements have been made reveal generally lower yolk carotenoid

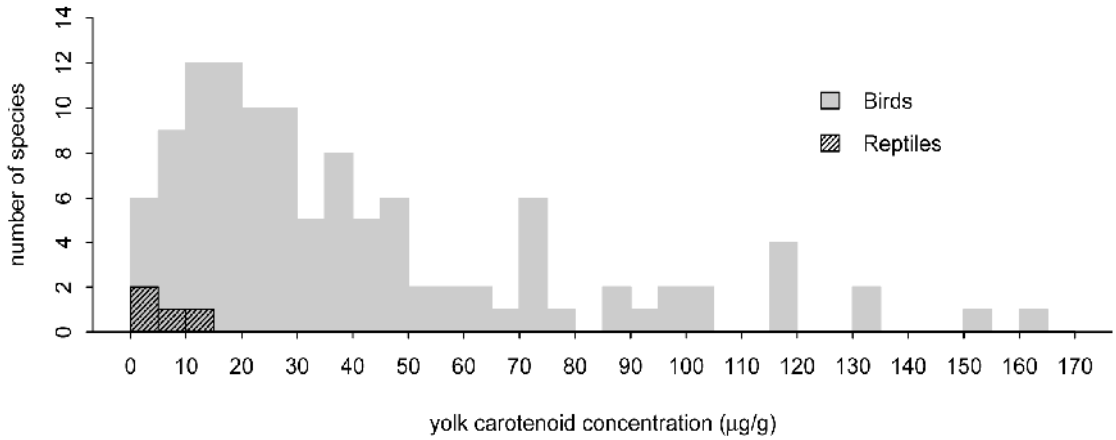



**Figure 2.4.** Inter-specific variation in the carotenoid concentrations of avian and reptilian eggs. Data for birds taken from Biard et al. (2009), *N* = 112 species. Data for reptiles taken from this study, Thompson et al. (1999), Speake et al. (2001) and Dierenfeld et al. (2002).

levels than is typical for avian eggs (**Figure 2.4**). Given the similar cleidoic egg structure but broadly different reproductive strategies of birds and reptiles, this taxonomic comparison may provide a useful basis for studying the determinants of carotenoid deposition in eggs. For example, the lower levels of carotenoids found in reptilian eggs may reflect constraints imposed by maternal fecundity, since reptiles tend to produce larger clutches than birds (Shine 2005), or may have an adaptive role. For example, avian embryos typically develop much faster than those of reptiles, with significantly higher metabolic rate and oxygen consumption (Vleck & Hoyt 1991; Ricklefs & Stark 1998), and may therefore require improved antioxidant defences to compensate for increased free-radical production and oxidative stress associated with rapid growth (Catoni et al 2008; Monaghan et al. 2009; but see **Chapter 5**).

In conclusion, this study has shown that while extraction of yolk carotenoids by LLE may be appropriate for species of birds with high carotenoid concentrations, it is less effective for HPLC analysis of carotenoids in species with low yolk carotenoid levels due to the co-recovery of large amounts of contaminating lipid. Such species may include some birds and many reptiles where carotenoids are present at low concentrations. In these cases, the use of SPE pre-treatment to further isolate carotenoids from yolk lipids can significantly improve chromatographic separations whilst achieving similar yields to standard LLE.

# Chapter 3 Proximate sources of variation in the antioxidant content of green turtle eggs: effects of maternal diet and laying order.

## 3.1 Abstract

Dietary antioxidants, such as vitamin E and carotenoids, are potentially limiting resources for egg production in animals and it has been suggested that females may allocate them adaptively within clutches. Variation in antioxidant provisioning to eggs may therefore reflect differences in maternal diet and/or non-dietary phenotypic differences among females. However, surprisingly little is known about how such factors contribute to variation in yolk antioxidant levels in wild populations, particularly in non-avian taxa. Similarly, within-female variation in antioxidant provisioning to eggs has only previously been studied in birds in the context of sibling rivalry and parental care, which limits general conclusions regarding the causes and adaptive significance of such trends. In this chapter, I compare within- and among-female variation in the provisioning of vitamin E and carotenoids to eggs in two populations of green turtles (*Chelonia mydas*), one consuming a natural diet (wild individuals) and the other a uniform captive diet (farmed individuals). Maternal diet influenced overall levels of antioxidant provisioning, as captive eggs contained significantly less carotenoids, but more vitamin E, compared to wild eggs. However, antioxidant levels in eggs varied considerably amongst captive females, despite an identical diet, and the degree of variation was not significantly different to that of the wild population. In both populations, yolk concentrations of antioxidants varied little within clutches, but varied markedly across successive clutches laid by individual females within a season. Vitamin E concentrations increased with clutch order whilst carotenoid levels declined, indicating that females did not consistently bias antioxidant investment to specific offspring. Overall, these results suggest that maternal diet influences antioxidant provisioning to eggs at the population level but is a relatively minor source of individual variation among female green turtles. Post-ingestion processes are therefore likely to underpin much of the variation in yolk antioxidant levels within populations. Females may be limited by the physiological availability of antioxidants for egg production – carotenoid levels declined in later clutches consistent with maternal constraints – however the increased provisioning of vitamin E over successive clutches suggests that active processes may regulate deposition of this antioxidant in eggs. A possible hormonal mechanism is discussed.

## 3.2 Introduction

In oviparous organisms, variation in egg size and composition can have profound effects on offspring phenotype and survival (Williams 1994; Mousseau & Fox 1998a); however the proximate mechanisms underlying this variation are still poorly understood. For example, a largely unresolved question is whether females are limited by the availability of specific nutrients, or whether non-resource-based, physiological mechanisms regulate maternal investment (reviewed in Williams 2005).

Egg production draws on numerous maternal reserves, and in addition to the energetic resources needed to support embryonic growth, females provision their eggs with a cocktail of developmentally important vitamins, minerals, hormones and immunoglobulins (Blount et al. 2002a; Groothuis et al. 2006). For example, avian and reptilian egg yolks contain significant quantities of lipophilic antioxidant compounds, including vitamin E and carotenoids, which are thought to have an adaptive function in protecting yolk lipids and embryonic tissues from oxidative damage (Blount et al. 2000; Surai 2002; but see Constantini & Møller 2008 and **Chapter 5**). Carotenoids are also potent immunomodulants and may facilitate maturation of the neonatal immune system (e.g. Saino et al. 2003). Vitamin E and carotenoids are only produced *de novo* in photosynthetic organisms meaning they are potentially limiting, dietary resources in animals (Grether et al. 1999; Hill et al. 2002; Catoni et al. 2008). However, some authors have argued that they are likely to be ubiquitous and non-limiting in the environment (Hudon 1994; Hadfield & Owens 2006),

Female birds vary markedly in the quantities of antioxidants that they deposit into eggs (e.g. Rubolini et al. 2006; Safran et al. 2008; Saino et al. 2008), although whether this variation is due to differential dietary access is equivocal. Experimental feeding studies have consistently shown that birds supplemented with vitamin E and carotenoids deposit more of these compounds into eggs (e.g. Blount et al. 2002a; Grobas et al. 2002; Royle et al. 2003; McGraw et al. 2005; Biard et al. 2005), establishing a potential link between maternal nutrition and egg quality. Under natural conditions, variation in yolk carotenoid levels has also been linked with the seasonal or habitat-related abundance of carotenoid-rich food items (Cassey et al. 2005; Hargitai et al. 2006; Török et al. 2007). However, whilst these studies have confirmed the potential for dietary limitation, they

have tended to focus on population-level shifts in antioxidant provisioning, rather than the effect of diet on individual variation *per se* (but see Grobas et al. 2002). In addition to dietary heterogeneity, there may be inherent physiological differences among females in their ability to incorporate dietary antioxidants into eggs. Multiple factors related to age, health, genotype, and neonatal nutrition are known to effect the efficiency with which lipid soluble antioxidants are absorbed and assimilated from the diet (reviewed in Surai 2002; Negro et al. 2001b; Blount et al. 2003; Hõrak et al. 2004), and may thus effect their availability for egg production. Indeed, in captive birds, substantial variation in yolk and plasma antioxidants prevails even when animals are maintained on a uniform diet (Bortolotti et al 1996; Negro et al. 2001b; Grobas et al. 2002), although how this compares to variation under natural conditions is not known. Thus, while there is broad consensus that both dietary and physiological factors may contribute to antioxidant deposition in eggs, their relative contributions in wild populations, and the physiological mechanisms involved, are poorly understood.

In birds, levels of vitamin E and carotenoids deposited in eggs also vary systematically across the laying sequence within clutches (Royle et al. 1999; Blount et al. 2002a), as do egg size and concentrations of many other key egg constituents (Slagsvold et al 1984; Groothuis et al. 2006). Such 'laying-order effects' have attracted considerable attention in evolutionary ecology as potentially adaptive, maternal strategies for manipulating offspring phenotypes (Slagsvold et al 1984; Royle et al. 1999; Blount et al. 2002a; Groothuis et al. 2005). For example, sequential egg-laying and hatching asynchrony are common in birds, and the monopolisation of parental care by older siblings can create hierarchies of offspring quality that favour earlier-laid chicks (Magrath 1990). By reducing their investment of antioxidants in later eggs, females may reinforce such hierarchies to facilitate adaptive brood reduction, or bias their investment of limiting resources to the more valuable core chicks (Royle et al. 1999, Royle et al. 2001; Hall et al. 2010). However, recently it has been argued that laying order effects may be an inevitable by-product of changes in maternal physiological state during egg production (Krist et al. 2004; Williams et al. 2005). If within-clutch variation in egg composition has an adaptive value for females, we should expect that patterns of investment will vary among taxa with different life histories and reproductive strategies. However, virtually nothing is known about within-clutch variation in antioxidant provisioning in non-avian taxa.

The aim of this study was to assess the importance of dietary heterogeneity and laying order as determinants of vitamin E and carotenoid provisioning in the eggs of green turtles (*Chelonia mydas*). To differentiate between dietary and post-ingestion sources of variation we compared antioxidant deposition in the eggs of two populations with contrasting maternal diets: a captive (farmed) population provided with a homogenous cereal-based diet that was identical for all individuals, and a wild population consuming a natural diet that consists of a diverse range of macroalgae and seagrass species (Bjorndal 1997; Hirth 1997). If differential acquisition of dietary antioxidants underpins variable provisioning in eggs, we should expect to find reduced variation among females when diet is standardised (as for carotenoid-based plumage colour in birds; McGraw & Hill 2001; Hadfield & Owens 2006). Female marine turtles produce multiple large clutches of eggs within a single breeding season, however very little is known of how females allocate key nutrients within and among clutches. Given the broad differences in reproductive strategies and life history between turtles and the majority of birds (large clutch size, multiple clutches within a season, lack of parental care, environmentally determined incubation conditions, low offspring survival), such data may provide a useful comparison for understanding the causes and functional relevance of within-female patterns of antioxidant provisioning to eggs.

## 3.3 Materials & Methods

### 3.3.1 Study sites and field procedures

**Wild turtles.** Eggs were collected at the time of oviposition from wild turtles nesting at Ascension Island, South Atlantic Ocean (14°20′W, 7°55′S) during the 2007 nesting season (January – June). In order to assess within-female variation in antioxidant provisioning across successive clutches, a randomly selected sample of females ($N$ = 20) nesting on Long Beach between 2nd January - 12th January were fitted with VHF radio transmitters (Biotrack Ltd., Wareham, UK), affixed to the carapace with a two-part epoxy resin. Less than 3 % of nesting activity at Ascension Is. occurs prior to January (Godley et al. 2001), so there is a high probability that tagged females were encountered whilst laying their first clutch. Females were also fitted with a PIT tag (Passive Integrated Transponder; Identichip, Animalcare Ltd., UK) implanted into the triceps muscle of the right fore-flipper to assist identification.

Nesting study females were re-located using a scanning VHF receiver (AOR, Derbyshire, UK) and YAGI antenna (Biotrack Ltd.) during nightly patrols of the nesting beach (20:00 – 05:00). Females laid 3 – 5 clutches (mean = 4.3 clutches female$^{-1}$) in the period from January – April when tracking was ended. While we cannot be certain that females did not nest again after this date, previous estimates have suggested a mean clutch frequency of 3 clutches female$^{-1}$ in this population (Mortimer & Carr 1987), so it is likely that we captured the majority of the laying season for our study animals. A single female was never recaptured (potentially due to tag loss or single nesting) and a number of clutches laid by study females could not be sampled (e.g. if females were encountered post-nesting or used alternative nesting sites), giving 67 clutches from 19 females for analysis of within-female variation.

Marine turtles lay large clutches (mean = 120 ± 27 eggs for this population), thus to effectively assess within-clutch variation in egg composition we sampled 3 eggs at approximately the 10$^{th}$, 50$^{th}$ and 10$^{th}$ from last (hereafter $n$ – 10$^{th}$) positions in the laying sequence for each clutch of our study females. Fresh eggs and separated egg components (yolk, albumen, shell) were weighed on an electronic balance (± 0.2g) and aliquots of the homogenised yolk stored at -45ºC for 3-5 months before transport on dry ice to our laboratory in the UK for analysis of antioxidant compounds. All study clutches were excavated post-hatching to determine clutch size from the number of shell fragments and unhatched eggs.

**Captive turtles.** Studies of captive turtles were conducted at the breeding population at Boatswain Bay, Cayman Islands during the 2008 nesting season (June – September). Breeding stock in this population consists of wild caught females from various Atlantic rookeries and their first generation offspring. Captive turtles were fed a pelleted, cereal-based diet (SouthFresh Feeds, Demopolis, AL) provided on a twice daily basis to the surface of the holding pond by farm staff (see Appendix **Figure A3.3**). Sufficient pellet was provided at each feeding to ensure all animals grazed continuously for c. 30 minutes, corresponding to a feed ration of approximately 1 % of maternal body mass per day. Biochemical analysis of the feed in our laboratory indicated a vitamin E content of 42.8 ± 1.4 mg kg$^{-1}$ and a carotenoid content of 6.6 ± 0.3 mg kg$^{-1}$ (mean ± SE from three separate batches; see below). Females had access to an artificial nesting beach, and nesting activity of a sample of females ($N$ = 18) was monitored on a nightly basis by farm staff over the course of the breeding season to locate successive clutches

of individuals. All individuals were fitted with PIT tags and/or unique flipper tags to allow identification. All but two individuals were observed to lay multiple clutches (mean = 3.2 clutches female$^{-1}$, range = 2 – 4 clutches female$^{-1}$), giving 47 clutches from 16 females for analysis of within female variation. Three eggs were sampled from each clutch of study females following the same protocol as used for wild turtles, and aliquots of homogenised yolk stored at -80 ºC (3 – 5 months) before transport on dry ice to the laboratory for antioxidant analysis. Clutch size was determined for each clutch of study females during relocation to artificial incubators. Data on the mass of yolk and albumen fractions was not collected for captive animals.

### 3.3.2 Biochemical analyses.

**Yolk antioxidants.** Concentrations of carotenoids in eggs of wild and captive turtles were analysed by HPLC following solid phase extraction on diol-silica cartridges as described in **Chapter 2**. Concentrations of vitamin E in yolk were also determined by HPLC following hydrolysis of bulk lipids using a modification of the alkaline saponification procedure described by Gaal et al. (1995). A 150-180 mg aliquot of frozen yolk was homogenised in 5 mL of ethanolic pyrogallol (5% w/v in ethanol), 0.7 mL of aqueous KOH (50% w/v) was immediately added, and the saponification mixture loosely stoppered under nitrogen gas and heated at 70 ºC for 30 min. After cooling, vitamin E was recovered with the addition of 5 mL of hexane and 10 mL of Millipure water, followed by gentle shaking for 2 min and centrifugation for 4 min at 1,500 rpm to separate the phases. A 3 mL portion of the upper organic phase was drawn off, evaporated to dryness on a rotary evaporator and vitamins reconstituted in 0.5 mL of ethanol ready for HPLC. Separation utilised a Spherisorb S3ODS2 3 µm $C_{18}$ reverse-phase HPLC column, 150 × 4.6 mm (Waters), with a mobile phase of 97:3 methanol/water (v/v) at an isocratic flow rate of 1.1 mL min$^{-1}$, and fluorimetric detection (295 nm excitation; 330 nm emission). Levels of α-tocopherol and γ-tocopherol were quantified relative to external standards prepared in ethanol. Recovery tests using exogenous standards indicated that > 95% of α-tocopherol survived saponification and repeatability was high (coefficient of variation for 5 replicates = 2.1%)

**Feed antioxidants.** The vitamin E and carotenoid content of the pelleted farm diet was determined using a similar saponification procedure as described for yolk samples (above). Briefly, ~ 250 µg of finely powdered feed (ground in a ball mill) was

rehydrated with 250 µl distilled water (10 mins) and then saponified with 0.5 ml of 50 % KOH and 5 ml ethanolic pyrogallol (5 % w/v) at 70 ºC for 15 minutes. The antioxidants were recovered with the addition of 5 ml hexane / diethyl ether (1:1 v/v) and 10 ml distilled water, followed by centrifugation at 1,500 × g to separate the phases. The organic phase was transferred to a separatory funnel and repeatedly rinsed with distilled water until neutral pH was reached. The organic phase was then evaporated under a gentle stream of nitrogen gas and the vitamin E and carotenoids reconstituted in methanol and quantified using HPLC as described for yolk samples (**Chapter 2** and see above)

**3.3.3 Statistical analyses.** In order to test the hypothesis that dietary access to antioxidants underpins variable deposition in eggs, I compared the relative variation in vitamin E and carotenoid concentrations among first-laid clutches of wild turtles with first-laid clutches of captive females maintained on a uniform diet (antioxidant concentrations were expressed as the mean of 3 eggs as within clutch variation was negligible; see Results). Tests of relative variation are independent of the mean, so can be used to compare the magnitude of variation in populations where absolute trait values differ (van Valen 2005). All comparisons were performed using Levene's test for homogeneity of relative variation (Schultz 1985; van Valen 2005). The test adapts the standard median Levene's test to the analysis of relative variation by expressing absolute deviations for each measurement as a ratio of the group median (median-ratio Levene's tests; *sensu* Schultz 1985) or by applying the standard test to log-transformed data (median-log Levene's test). Both variants are robust under non-normality and skewness in the data (Schultz 1985). I present results from median-ratio tests, however the findings are qualitatively unchanged using the median-log form.

Within-female variation in yolk antioxidant levels for captive and wild turtles was analysed using hierarchical mixed-effects models with a nested error structure (egg position nested within clutch nested within female). Proportions of total variation explained at each level of the hierarchy were estimated by variance component analysis of restricted maximum likelihood (REML) models (following Crawley 2007). The significance of the variance component explained by female identity was evaluated using likelihood ratio tests after deletion from the REML fit model and rescaling of the error structure with clutch as the highest level in the hierarchy. Egg position in the laying sequence (3 level factor), clutch number (4/5 level factor), clutch size (covariate)

and two-way interactions between them were modelled as fixed effects, and significance assessed by stepwise deletion of non-significant predictors from maximum likelihood models ($\alpha = 0.05$), starting with interaction terms. Where a significant effect was found, differences between factor levels were assessed using post-hoc Tukey tests in the multcomp package for mixed-effects models (Hothorn et al. 2008). Means are presented as ± 1 SE and all statistical tests are two tailed. All analyses were carried out using the R 2.9.2 statistical package (R Development Core Team 2009).

## 3.4 Results

### 3.4.1 Maternal diet

In comparison to wild females, first-laid clutches of captive turtles contained higher levels of vitamin E (Welch's *t* test, $t = 3.1$, d.f. = 32, $p = 0.004$), but considerably lower levels of carotenoids ($t = 10.7$, d.f. = 19.1, $p < 0.001$; **Table 3.1**). However, concentrations of vitamin E and carotenoids varied considerably among first-laid clutches of captive females, despite a uniform maternal diet (2-fold and 3-fold respectively; **Table 3.1**). Moreover, the relative variation among captive females (relative to the median; see § 3.3.3) was not significantly different than the relative variation among wild females (median-ratio Levene's test; **Table 3.2**), suggesting that dietary heterogeneity is a minor source of variation in antioxidant provisioning to eggs under natural conditions. Despite significant variation among females, concentrations of vitamin E and carotenoids in first-laid clutches were not significantly correlated for either captive (Pearson's correlation, $r = 0.15$, $n = 18$, $p = 0.54$; estimate: 0.005 ± 0.01) or wild turtles ($r = 0.30$, $n = 20$, $p = 0.19$; estimate: - 0.07 ± 0.05).

**Table 3.1.** Concentrations of vitamin E and carotenoids in eggs from first laid clutches of wild ($N = 20$) and captive green turtles ($N = 18$). Concentrations are expressed in $\mu g\ g^{-1}$ of fresh yolk.

| | Wild | | Captive | |
|---|---|---|---|---|
| Antioxidant | mean | range | mean | range |
| Carotenoids | 5.14 ± 0.4 | 2.0 – 8.3 | 0.84 ± 0.07 | 0.4 – 1.4 |
| Vitamin E | 32.2 ± 1.7 | 14.9 – 48.0 | 41.0 ± 2.2 | 27.5 – 61.1 |

**Table 3.2.** A comparison of the relative variation in vitamin E and carotenoid concentrations in eggs from the first-laid clutches of wild and captive green turtles.

| | coefficient of variation (%) | | median-ratio Levene's test | |
|---|---|---|---|---|
| antioxidant | wild | captive | $F_{1,37}$ | $p$ |
| carotenoids | 33.5 | 34.2 | 0.26 | 0.61 |
| vitamin E | 24.2 | 22.3 | 0.33 | 0.57 |

### 3.4.2. Laying order

In an analysis of antioxidant provisioning within and among successive clutches, differences among females accounted for a majority of the variation in carotenoid and vitamin E concentrations in both wild and captive populations (hierarchical GLMM; among female variance component > 68 %; **Table 3.3**). However, there was also significant variation among successive clutches laid by individual females (among clutch variance component = 12 – 27%; **Table 3.3**): in both populations concentrations of vitamin E increased over successive clutches whereas carotenoid levels declined (**Figures 3.1C** and **3.1D**). The relative decrease in carotenoid concentrations between consecutive clutches was not significantly predicted by the relative increase in vitamin E concentrations in either population (GLMM with female as a random factor, wild: $\chi_1^2$

**Table 3.3.** Sources of variation in vitamin E and carotenoid concentrations within and among successive clutches of individual green turtles from a hierarchical GLMM (egg position nested in clutch number nested in female).

| | female | | clutch number | | egg position | |
|---|---|---|---|---|---|---|
| antioxidant | $\chi_1^2$ | $p$ | $\chi_4^2$ | $p$ | $\chi_2^2$ | $p$ |
| | | | wild turtles | | | |
| carotenoids | 38.0 | < 0.001*** | 70.0 | < 0.001*** | 2.3 | 0.31 |
| vitamin E | 29.7 | < 0.001*** | 56.6 | < 0.001*** | 0.55 | 0.76 |
| | | | captive turtles | | | |
| carotenoids | 50.4 | < 0.001*** | 23.9 | < 0.001*** | 1.80 | 0.41 |
| vitamin E | 19.0 | < 0.001*** | 12.4 | 0.006 ** | 2.46 | 0.29 |

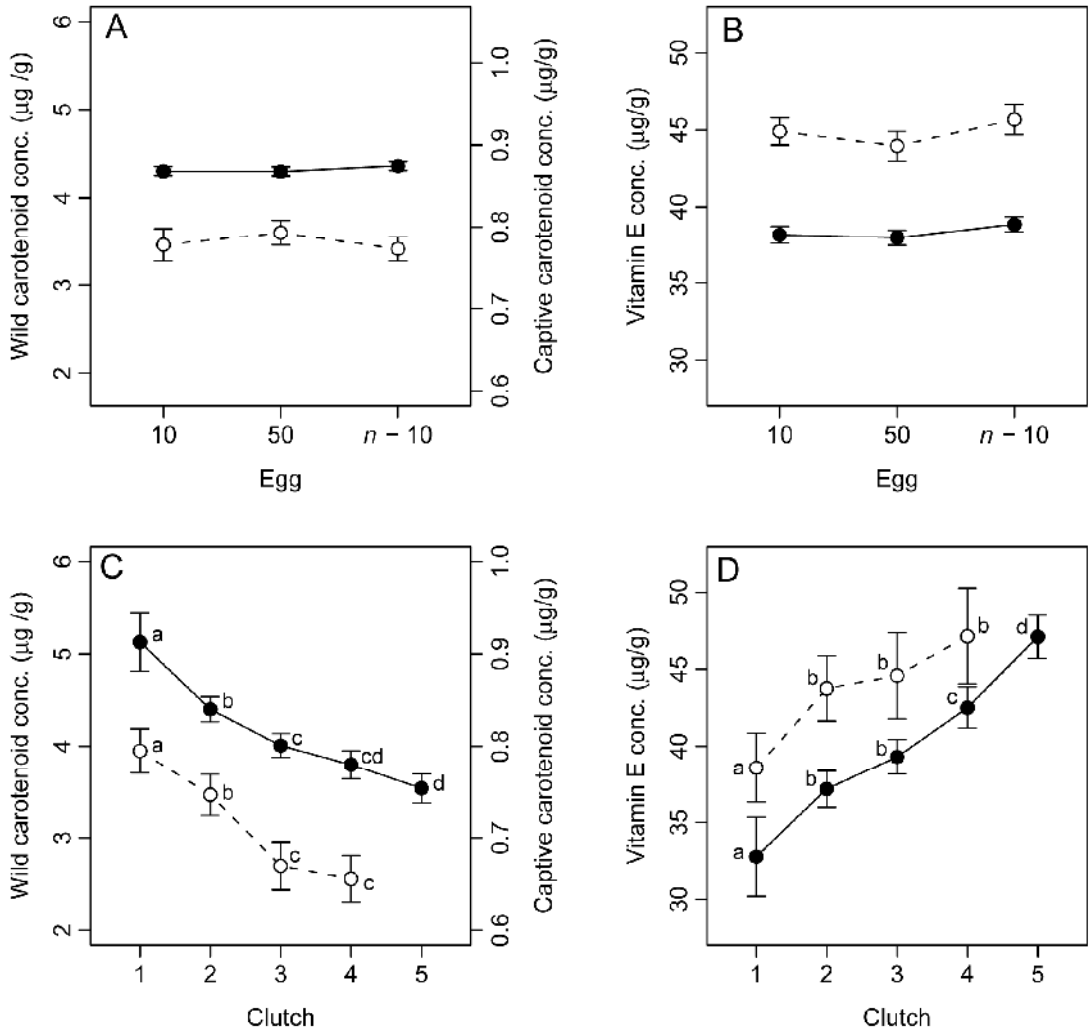



**Figure 3.1.** Variation in yolk antioxidant concentrations within and among successive clutches of individual wild (●) and captive (○) green turtles. (A) carotenoid concentrations within clutches; (B) vitamin E concentration within clutches; (C) carotenoid concentrations among clutches; (D) vitamin E concentrations among clutches. Estimates ± 1 SE from hierarchical GLMMs are shown (egg nested within clutch nested within female). Egg *n* – 10 in panels A and B refers to the 10th egg from last in the laying sequence. Unshared letters (a - d) in panels C and D denote significant differences between clutches in multiple comparison Tukey tests ($p < 0.05$).

= 0.10, $p = 0.76$, estimate = - 0.03 ± 0.12; captive: $\chi^2_1 = 0.45$, $p = 0.50$, estimate = - 0.05 ± 0.08), suggesting that these trends were not causally related. In both populations clutch size also increased with clutch order (GLMM, wild: $\chi^2_4 = 15.2$, $p = 0.004$; captive: $\chi^2_3 = 19.4$, $p < 0.001$), although this was as a result of first-laid clutches being smaller than all subsequent clutches (**Figure 3.2**). After controlling for clutch order, eggs from larger clutches contained lower concentrations of carotenoids in the wild population (GLMM, $\chi^2_1 = 6.6$, $p = 0.01$; estimate = - 0.01 ± 0.004), but not in the

captive population ( $\chi_1^2 = 0.15$, $p = 0.70$; estimate = - 0.0002 ± 0.0005). Clutch size did not significantly effect concentrations of vitamin E deposited in eggs in either population (GLMM, both $\chi_1^2 < 0.40$, $p > 0.50$).

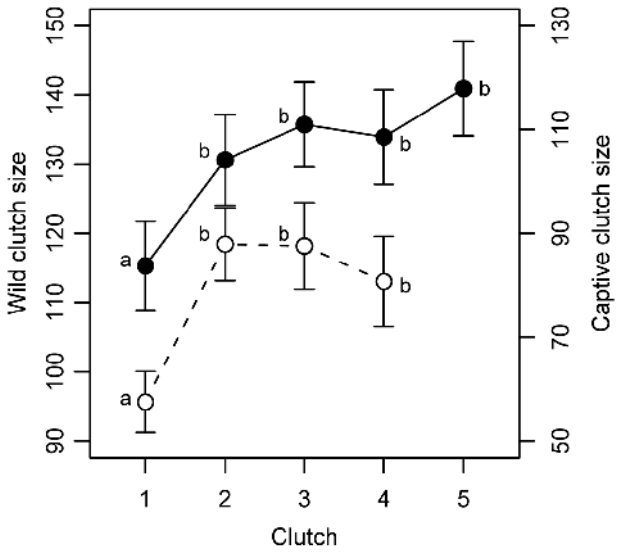



**Figure 3.2.** Variation in clutch size across successive clutches of individual wild (●) and captive (○) green turtles.

Within clutches there was very little variation in yolk antioxidant concentrations across the laying sequence in either population (within-clutch variance component < 5 %; **Table 3.3; Figures 3.1A** and **3.1B**). Thus a single egg was generally representative of the whole clutch in terms of antioxidant content. In contrast, masses of eggs and yolks did not vary systematically with clutch order (hierarchical GLMM, all $\chi_4^2 < 7.0$, $p > 0.15$) but were strongly predicted by the position of eggs within the laying sequence of individual clutches (all $\chi_4^2 > 25.0$, $p < 0.001$). Eggs in the centre of a clutch were heavier in comparison to those in peripheral positions, while yolk mass declined with laying sequence and was significantly lower in later eggs.

## 3.5 Discussion

Variation in the provisioning of vitamin E and carotenoids to eggs has been shown to influence numerous aspects of offspring phenotypic quality and may therefore be an important source of maternal effects (Surai 2002; Biard et al. 2005; McGraw et al. 2005; Saino et al. 2008; Ewen et al. 2009; but see **Chapter 5**); however, the proximate basis for such variation in wild populations is not well understood. This chapter investigated how maternal diet and laying order contributes to variable deposition of vitamin E and carotenoids in eggs in two populations of green turtles consuming either a natural diet (wild population) or a uniform captive diet (farmed population).

### 3.5.1 Maternal diet

To date, much of the evidence that carotenoids are a limiting, dietary resource under natural conditions derives from population-level relationships between the geographical and seasonal abundance of carotenoid-rich food sources, and concentrations of carotenoids deposited in egg yolks or colourful integument (Grether et al. 1999; Cassey et al. 2005; Hargitai et al. 2006; Török et al. 2007; Sternalski et al. 2009). Supplemental feeding experiments have also confirmed a link between levels of carotenoids and vitamin E in the maternal diet and levels deposited in eggs (Blount et al. 2002a; Grobas et al. 2002; Royle et al. 2003; McGraw et al. 2005; Biard et al. 2005). Consistent with these previous studies, maternal diet significantly influenced population mean levels of antioxidants in green turtle eggs, as captive females fed a cereal-based diet deposited considerably lower concentrations of carotenoids, but more vitamin E, in egg yolks compared to wild females feeding predominantly on marine macrophytes (Bjorndal 1997; Hirth 1997).

However, the determinants of population (or treatment) means for a given trait often tell us little about the underlying causes of inter-individual variation (Bennett 1987). A clear prediction of the dietary limitation hypothesis is that among-female variation in antioxidant provisioning to eggs should be reduced when diet is standardised for all individuals (e.g. McGraw & Hill 2001; Hadfield & Owens 2006). On the contrary, we found considerable variation in yolk levels of vitamin E and carotenoids among captive females which was not significantly different to the variation found among wild females. This suggests that differential dietary access to antioxidants is a relatively minor source of variation in egg provisioning in wild turtles. To our knowledge only two studies have explicitly shown that individuals vary in their dietary acquisition of carotenoids in wild populations (Bortolotti et al. 2000; Hill et al. 2002), and one of these was in carnivorous birds with very heterogeneous and carotenoid-deficient diets (Bortolotti et al. 2000). In contrast, vitamin E and carotenoids occur at high levels in green plant tissues (Holden et al. 1999; Ortiz et al. 2006) and are likely to be ubiquitous and non-limiting resources in the herbivorous diet of wild green turtles.

Overall, these results suggest that dietary access to antioxidants influences provisioning to eggs at the population level, but is a relatively minor source of variation among

individual females. Studies in birds have similarly shown that dietary supplementation with vitamin E and carotenoids increases their deposition in egg yolk and plumage (respectively) without reducing inter-individual variation in these traits (McGraw and Hill 2001; Grobas et al. 2002; Hadfield and Owens 2006). Thus, post-ingestion processes likely account for much of the intra-population variation in yolk antioxidant levels. Various phenotypic factors are known to affect how efficiently animals absorb and assimilate dietary vitamin E and carotenoids, and could therefore affect their availability for egg production (e.g. Blount et al. 2003; Hõrak et al. 2004). However, based on the results of this study, two lines of evidence suggest that different physiological pathways regulate the provisioning of vitamin E and carotenoids in green turtle eggs (see also **Chapter 4**):

1) Vitamin E and carotenoid concentrations in yolk were not significantly correlated in either study population, even among captive females where relative dietary intakes were identical for all individuals. Both of these micronutrients are absorbed, transported and deposited into egg yolk as components of lipoproteins (reviewed in Rock et al. 1996; Surai 2002). Thus, if individual variation in gut function and/or lipoprotein metabolism influenced the provisioning of vitamin E and carotenoids in eggs, we would expect yolk concentrations to be positively correlated.

2) Changes in vitamin E and carotenoid concentrations across successive clutches of individual females were antithetical in both wild and captive populations: vitamin E provisioning increased with clutch order while carotenoid levels declined (**Figures 3.1C** and **3.1D**). These opposing trends are unlikely to be causally related (e.g. by competitive exclusion from lipoproteins) as the decrease in yolk carotenoid concentrations between consecutive clutches was not significantly related to the corresponding increase in yolk vitamin E. Separate mechanisms must therefore underpin intra-individual variation in vitamin E and carotenoid provisioning in eggs and are discussed further in Section 3.5.2.

### 3.5.2. Laying order

Variation in egg size and composition within clutches is typical in birds (Slagsvold et al. 1984; Royle et al. 1999; Blount et al. 2002; Groothuis et al. 2006). In particular, concentrations of vitamin E and carotenoids in yolk have been found to decrease

significantly between the first and last-laid eggs of a clutch (Royle et al. 1999; Blount et al. 2002a; Hõrak et al. 2002; Cassey et al. 2005; Williamson et al. 2006; Groothuis et al. 2006; but see Török et al. 2007). Unlike birds, antioxidant concentrations in green turtle eggs did not vary systematically across the laying sequence of individual clutches; however, there was marked variation in vitamin E and carotenoid levels among successive clutches laid by individual females (**Figure 3.1**). This finding is intuitive given the contrasting reproductive physiologies of birds, where yolk follicles mature and ovulate sequentially (Johnson 2000), and reptiles, where populations of follicles contributing to each clutch are developed and ovulated simultaneously (e.g. Callard et al. 1978; Licht et al 1982). A complete reptilian clutch may therefore be analogous to a single avian egg with respect to yolk formation and composition. Janzen et al. (1998) and Bowden et al. (2000) similarly found little variation in yolk steroid hormone levels within clutches of freshwater turtles compared to significant variation among clutches. The low within-clutch variance in egg composition in reptiles may make them a useful system for studying egg-mediated maternal effects, as offspring phenotypes can be reliably related to the composition of eggs sampled from the same clutch (see **Chapter 5**), which is often not possible in birds.

Studies in birds have suggested that within-clutch patterns of antioxidant provisioning may comprise an adaptive maternal strategy for allocating limiting resources among offspring and may facilitate brood size reduction (Royle et al. 1999; Blount et al. 2002a; Hõrak et al. 2002). However, sea turtles lack post-laying parental care and there is no obvious hierarchy in the reproductive value of successive clutches. Indeed, in the present study, yolk levels of vitamin E and carotenoids varied antithetically with clutch order (carotenoid levels decreased while vitamin E increased; **Figure 3.1**), indicating that females did not consistently bias their provisioning of antioxidants to specific offspring. Rather, these results suggest that intra-individual variation in antioxidant provisioning may be a by-product of changes in maternal physiological state during egg production (see Krist et al 2004 and Williams et al. 2005 for similar arguments).

The decline in yolk carotenoid levels which we observed across successive clutches of wild and captive green turtles (**Figure 3.1C**) is consistent with decreases across the laying sequence described in many birds (Royle et al. 1999; Hõrak et al. 2002; Cassey et al. 2005; Williamson et al. 2006), suggesting that it is a phylogenetically conserved feature of egg production. Indeed, it is difficult to envisage a shared aspect of life

history which would select for adaptive reductions in carotenoid investment with laying order in both birds and sea turtles (e.g. brood size reduction hypotheses are not relevant in sea turtles; *sensu* Royle et al. 1999; 2001). The most parsimonious explanation for these trends is that yolk carotenoid levels reflect endogenous reserves in the female which are progressively depleted during egg production (see also Groothuis et al. 2006). Female birds are known to store large quantities of carotenoids in the liver and adipose tissue which are mobilised into the bloodstream during egg production (Negro et al. 2001a; McGraw & Toomey 2010). Depletion of this circulating pool of carotenoids during the maturation of successive eggs/clutches is likely to produce the observed declines in yolk carotenoid levels irrespective of the initial size of maternal reserves. Indeed, identical among-clutch trends were found in both wild and captive populations of green turtles despite marked differences in overall levels of carotenoid provisioning (**Figure 3.1C**).

The vitamin E content of eggs has also been shown to decrease with laying sequence in many species of birds, which is contrary to the significant increase in vitamin E levels across successive clutches of green turtles (**Figure 3.1D**). One possible explanation for this discrepancy is that intra-individual variation in yolk vitamin E levels is generated by endocrine cycles in the female (e.g. Williamson et al 2006). Oestrogen plays a pivotal role in regulating egg production in birds and reptiles (Licht et al. 1982; Johnson 2000), and has been shown to induce the secretion of vitamin E from the liver into the maternal bloodstream (Halifeoglu et al. 2003). Plasma oestrogen levels increase throughout yolk formation in sea turtles, reaching a maximum at the onset of nesting, by which time the full complement of pre-ovulatory follicles for all the clutches to be laid in a season are present (Rostal et al. 1998). Since variation in plasma vitamin E levels across the reproductive cycle has been shown to mirror the production of estradiol in reptiles (Lance et al. 1983), follicles destined for later clutches may develop with higher concentrations of vitamin E in maternal circulation and become enriched compared with earlier clutches. Clutch size is also known to be oestrogen-dependent in reptiles (Jones et al. 1975) and was larger for later clutches (**Figure 3.2**), consistent with an increase in maternal oestrogen levels during the formation of successive clutches.

Conversely, in birds, maternal oestrogen levels decline rapidly following ovulation of the first egg while subsequent follicles are still undergoing development (Williams et al. 2004), and may lead to decreased vitamin E provisioning with laying order. Thus, the

simultaneous declines in vitamin E and carotenoid provisioning across the laying sequence which are typical in birds may reflect different causal mechanisms (variation in maternal oestrogen levels and depletion of maternal reserves) operating in the same direction. If experimentally proven, hormonal regulation of vitamin E deposition in eggs would provide a potential mechanism by which females could actively adjust the antioxidant defences of their offspring. However, within females, changes in circulating oestrogen levels during egg production are likely to be relatively inflexible as they are governed by patterns of ovulation of yolk follicles (the source of the hormone; Rostal et al. 1998; Williams et al. 2004). Thus intra-individual variation in vitamin E provisioning may reflect a fixed constraint of egg production.

Overall, this study suggests that maternal diet is a relatively minor source of variation in antioxidant provisioning to eggs in wild green turtles, and that inherent physiological differences among females may be the principle determinant of vitamin E and carotenoid levels in eggs. While the physiological mechanisms involved require further investigation, yolk levels of vitamin E and carotenoids appear to be regulated independently (see also **Chapter 4**). Females may be limited by the post-consumption availability of carotenoids, linked to health status or other phenotypic factors (e.g. Blount et al. 2003; Hõrak et al. 2004); however non-resource-based constraints imposed by reproductive hormones may be an important source of variation in vitamin E provisioning and warrant further study. Whether females adaptively adjust the provisioning of antioxidants to their eggs to manipulate offspring phenotype is unclear (see **Chapter 5**); however, at the intra-individual level, this study suggests that variable antioxidant investment is likely to reflect constraints on egg production rather than constituting an adaptive maternal effect.

## 3.6 Appendix

*Photograph © W. Mustin, with permission.*

**Figure A3.3.** Photograph illustrating the rearing conditions and feeding protocol of captive green turtles at Boatswain Bay, Cayman Islands.

# Chapter 4 Relationships between maternal plasma antioxidants and concentrations in the eggs and hatchlings of wild green turtles.

## 4.1 Abstract

In all vertebrate species, mothers transfer essential fat-soluble micronutrients and antioxidants to their offspring via placental transmission or provisioning in eggs; however the factors which regulate such investment are generally poorly understood. At a proximate level, provisioning of fat-soluble antioxidants to eggs (and therefore offspring) may simply reflect variation in maternal plasma antioxidant status during egg production. In the following chapter I investigate how variation in maternal plasma levels of vitamin E and carotenoids relates to provisioning in eggs in wild green turtles and whether such variation is quantitatively transferred to their offspring. Carotenoid concentrations in egg yolk were strongly and positively predicted by maternal plasma concentrations, although lutein was preferentially incorporated into eggs in comparison to other plasma carotenoids. In contrast, yolk levels of vitamin E were unrelated to maternal plasma concentrations, suggesting that different factors regulate vitamin E and carotenoid deposition in eggs. Different isomers of vitamin E were transferred non-selectively from maternal plasma to yolk and hatchlings. Yolk concentrations of all fat-soluble antioxidants were strongly correlated with their respective concentrations in hatchling blood plasma, indicating that egg composition is an important determinant of antioxidant reserves during early life in this species. Overall, this study suggests that variable maternal deposition of antioxidants in eggs has the potential to influence offspring antioxidant status, but that the mechanisms underlying such variation are distinct for vitamin E and carotenoids (see also **Chapter 3**). I propose that egg carotenoid levels vary as a direct function of maternal body reserves, while maternal investments of vitamins E may be determined by transient, hormonally-induced increases in circulating vitamin concentrations during yolk formation - a process which is complete prior to arrival at the nesting grounds in sea turtles.

## 4.2 Introduction

Vitamin E and carotenoids are dietary micronutrients with essential regulatory and protective functions in animals (Debier & Larondelle 2005; Rock et al. 1996). Many of these roles may be of particular importance during early life stages: for example, vitamin E forms an integral part of the antioxidant system that protects embryonic tissues from free-radical induced oxidative stress (Surai 2002; Debier & Larondelle 2005; but see **Chapter 5**); while carotenoids have been linked with a range of health-related benefits in animals, including antioxidant activity, enhanced immunity and parasite resistance, all of which may be of particular relevance in neonates (Blount et al. 2000; Surai 2002; Saino et al. 2003; Ewen et al. 2009). Mothers must therefore ensure that their offspring are adequately provisioned with vitamin E and carotenoids, through placental transmission and milk in mammals (Debier and Larondelle 2005), and via the lipid-rich yolk of eggs in oviparous species (Blount et al. 2000; Surai 2002).

Nonetheless, studies in birds and reptiles have revealed a remarkable level of intra-specific variation in the quantities of lipophilic antioxidants transferred into eggs which remains largely unexplained (**Chapter 3**). Maternal nutrition has been shown to influence the provisioning of vitamin E and carotenoids in eggs (Blount et al. 2002a; Royle et al. 2003; McGraw et al. 2005; Biard et al. 2005); however the observation that individual variation within populations is not reduced when females are maintained on a uniform diet or provided with vitamin supplements (**Chapter 3**; Grobas et al. 2002) suggests that physiological factors rather than dietary constraints may often predominate. An improved understanding of the proximate physiological mechanisms which regulate antioxidant levels in eggs is therefore required.

Vitamin E and carotenoids contained in egg yolk are extracted from the maternal bloodstream during oocyte growth as components of plasma lipoproteins (Surai 2002). As the embryo develops, yolk antioxidants are then progressively transferred to embryonic tissues via the yolk sac membrane (Gaal et al. 1995). Variation in maternal plasma antioxidant levels during yolk formation may therefore be an important determinant of both yolk composition (Bortolotti et al. 2003) and offspring antioxidant status (Lin et al. 2005). However, maternal blood plasma represents the proximate source of resources available for egg production irrespective of ultimate mechanisms. For example, plasma antioxidant levels may be determined by endogenous reserves in

the female (Kardinaal et al. 1995; McGraw & Toomey 2010), or may reflect transient physiological states associated with yolk formation (Lance et al. 1983). Plasma concentrations of many yolk precursors increase dramatically during follicular growth in response to oestrogen induction (including lipophilic antioxidants; Lance et al. 1983; Halifeoglu et al. 2003), and the magnitude of this increase may be unrelated to maternal nutritional status (Christians & Williams 2001a). Consequently, relationships between levels of vitamin E and carotenoids in yolk and maternal plasma at the time when yolks are formed tell us little about the underlying sources of variation in antioxidant provisioning. Distinguishing between alternative sources of variation may therefore benefit from relating yolk antioxidants to baseline plasma levels in non-reproductive females.

The transfer of fat-soluble antioxidants to eggs may also be shaped by the selective deposition of only specific carotenoids and forms of vitamin E in yolk (Blount et al. 2002b; Royle et al. 2003). Carotenoids are a large and diverse family of compounds (> 700; Britton et al. 2004), while vitamin E encompasses 8 naturally occurring tocochromanols (4 tocopherols and 4 tocotrienols; Surai 2002). Female birds routinely transfer certain dietary carotenoids and tocochromanols into eggs more efficiently than others, suggesting a level of physiological discrimination (Hencken 1992; Surai & Sparks 2001; Blount et al. 2002b; Royle et al. 2003). Since different tocopherols/tocotrienols and carotenoids vary in their effectiveness as antioxidants and may have specific roles in tissues (Edge et al. 1997; Surai 2002), such ‘preferences’ may serve to optimise the nutritional balance of eggs for offspring (Blount et al. 2002b; Royle et al. 2003). However, yolk profiles may equally reflect general metabolic processes in the female. The preferential accumulation of specific carotenoids and tocopherols is common in animals and may be achieved through differential absorption of dietary micronutrients and processing in the liver (Parker 1996; Surai 2002), or through selective uptake from the bloodstream by certain tissues (e.g. feather and integument carotenoids in birds; McGraw et al. 2003a; McGraw & Toomey 2010). Since few studies have directly compared the antioxidant profiles in egg yolk with maternal plasma, we lack evidence that post-absorptive mechanisms exist to specifically adjust egg composition.

The aim of this study was to assess the quantitative transfer of vitamin E and carotenoids from the blood plasma of female green turtles (*Chelonia mydas*) to their

eggs and offspring. Female sea turtles lay multiple clutches within a season, however the complete set of pre-ovulatory follicles ('yolks') for all clutches are formed before the commencement of mating and nesting (Rostal et al. 1997; Rostal et al. 1998). Thus, nesting females are in a 'post-vitellogenic' condition, as evidenced by the return of plasma oestrogen and yolk precursors to baseline levels (Rostal et al. 1998). My specific objectives were to ascertain 1) whether lipid soluble antioxidant deposition in eggs is predicted by maternal plasma levels at nesting, 2) whether yolk antioxidants are quantitatively transferred to hatchlings, and 3) the extent to which specific carotenoids and tocopherols are discriminated during transfer to eggs and hatchlings.

## 4.3 Materials & Methods

**4.3.1 Sample collection and field procedures.** The study was conducted during two consecutive nesting seasons (January – June 2007 and 2008) at Ascension Island, South Atlantic Ocean. Transfer of fat-soluble antioxidants from egg yolk to hatchlings was assessed during the 2007 season, using a randomly selected sample of clutches laid between 17$^{th}$ January and 30$^{th}$ March ($N$ =20). Yolk antioxidant concentrations were determined from a sample of 3 eggs collected at oviposition from early, middle and late in the laying sequence of each clutch (10$^{th}$, 50$^{th}$ and 10$^{th}$ from last positions; see below). Yolks were separated from whole egg contents within 2 hours of laying using a domestic egg separator, and aliquots of the homogenised yolk stored at – 45 ºC (4-5 months) before transfer on dry ice to laboratory storage at – 80 ºC. Females were allowed to cover clutches naturally and nest sites were marked and fitted with surface cages from day 45 of incubation to trap emergent hatchlings. For a randomly selected sample of hatchlings from each clutch ($N$ = 5) I collected ~ 50 µl blood from the dorsal cervical sinus using 8mm × 30G syringes (BD Micro-Fine, Becton-Dickinson, NJ, USA) for analysis of plasma antioxidant levels (see § 4.3.2). Blood was immediately fractionated by centrifugation at 10,000 × g for 5 minutes and the plasma layer recovered and stored as described for yolks.

During the 2008 nesting season, blood and eggs were sampled from nesting females ($N$ = 34) to assess quantitative relationships between maternal plasma antioxidant concentrations and provisioning in eggs. Female sea turtles lay multiple clutches within a nesting season and the relationships between plasma and yolk micronutrients may vary over the laying cycle (as in birds: Bortolotti et al. 2003). Thus, to account for

potential seasonal effects, sampling was conducted in 3 discrete blocks corresponding to "early", "peak" and "late" stages of the nesting season (early: 15$^{th}$ – 25$^{th}$ Feb, $N$ = 11; peak: 25$^{th}$ March – 5$^{th}$ April, $N$ =12; late: 8$^{th}$ – 18$^{th}$ May, $N$ = 11; see Godley et al. 2001). Blood was collected during the quiescent stage of oviposition using the interdigitary vessel (IDV) technique described by Wallace & George (2007), as modified for green turtles (see Appendix Figure A4.3). Approximately 1-2 ml of blood was drawn into a 6 ml lithium heparin coated Vacutainer® tube (Becton-Dickinson) fitted with a 1" × 22 gauge needle and fractionated by centrifugation at 10,000 × g within 30 minutes of collection. The plasma layer was then recovered for micronutrient analysis. Yolk micronutrient levels were determined for a single egg sampled from approximately the middle of each clutch (~ 50$^{th}$ egg), as data collected in 2007 indicated low levels of within-clutch variation across the laying sequence (see § 4.3.3; also **Chapter 3**). Preparation and storage of yolk and plasma samples were as described above.

**4.3.2 Biochemical analyses.** Levels of carotenoids and vitamin E in egg yolk were analysed using high-performance liquid chromatography (HPLC) as described in **Chapters 2 and 3**. Vitamin E and carotenoids were extracted from blood plasma using previously described methods (Blount et al. 2003) and quantified using HPLC. For the analysis of maternal plasma, a 50 µl aliquot of plasma was vortex mixed with 100 µl ethanol (20 s) and 50 µl of 5% NaCl (w/v) and the fat-soluble micronutrients extracted into 500 µl hexane by vortex mixing (20 s) followed by centrifugation at 10,000 × g for 2 minutes. Similar methodology was used in the analysis of hatchling plasma, although a 10 µl aliquot of plasma was initially combined with 10 µl of 5% NaCl and 20 µl ethanol, and micronutrients extracted into 200 µl of hexane. A portion of the hexane phase was then recovered and evaporated to dryness on a rotary evaporator, and the sample reconstituted in 100 µl methanol and submitted to HPLC (HPLC conditions were as described in **Chapters 2 and 3**; low lipid levels in plasma extracts mean initial sample cleanup by solid phase extraction/saponification is not required). Instrument calibration and peak assignment were performed using standard solutions of tocopherols and tocotrienols in ethanol and standard solutions of carotenoids in methanol (lutein, zeaxanthin; Sigma-Aldrich Chemical Co.). Only α- and γ-tocopherol were identified in yolk and plasma samples.

**4.3.3 Statistical analysis.** I have previously demonstrated that fat-soluble antioxidant levels in eggs vary little within green turtle clutches (**Chapter 3**). Similarly, proportions of different carotenoids (lutein / zeaxanthin) and forms of vitamin E (α- / γ-tocopherol) in yolk did not vary systematically with laying order (GLMM with clutch as a random factor; all $\chi^2_2 < 2.0$, all $p > 0.35$) and within-clutch variation was negligible (within-subjects variance component < 1 %). Thus, for the purposes of the present study micronutrient levels in eggs were calculated as the average of 3 eggs. Standard parametric tests were used for all analyses (further details are provided in Results § 4.4). Plasma and yolk concentrations of fat-soluble antioxidants were tested for normality using Shapiro-Wilkes tests and found to fit a normal distribution ($p > 0.05$). Relative levels of different carotenoids and forms of vitamin E (expressed as proportions of the total) were arcsine square-root transformed prior to analysis. To account for seasonal variation in the relationship between maternal plasma and yolk antioxidants, stage of the nesting season was included as a factor in all models (see § 4.3.1). All analyses were performed using the R 2.9.2 statistical package (R Core Development Team 2009).

## 4.4 Results

Concentrations of lipid-soluble antioxidants in maternal blood plasma varied considerably among individuals and are summarised in **Table 4.1**. Yolk carotenoid levels were strongly predicted by carotenoid levels in maternal plasma (ANCOVA with stage of nesting season as a factor, see § 4.3.1; $F_{1,31} = 82.5$, $p < 0.001$; **Figure 4.1A**); however there was no relationship between concentrations of vitamin E in maternal plasma and concentrations deposited in yolk ($F_{1,31} = 1.7$, $p = 0.2$; **Figure 4.1B**), suggesting that carotenoid and vitamin E levels in eggs may be regulated by different

**Table 4.1.** Concentrations of fat-soluble antioxidants in the blood plasma of nesting green turtles and newly emerged hatchlings. Hatchling plasma values are mean of $N = 5$ individuals per clutch from $N = 20$ clutches. Values are expressed in μg $ml^{-1}$ plasma.

| Antioxidant | Maternal plasma | | Hatchling plasma | |
|---|---|---|---|---|
| | mean ± SE | range | mean ± SE | range |
| Vitamin E | 11.6 ± 0.7 | 5.3 – 26.0 | 53.2 ± 3.3 | 29.9 – 85.6 |
| Carotenoids | 0.75 ± 0.05 | 0.20 – 1.40 | 3.27 ± 0.2 | 1.5 – 5.1 |
| | $N = 34$ | | $N = 100$ | |

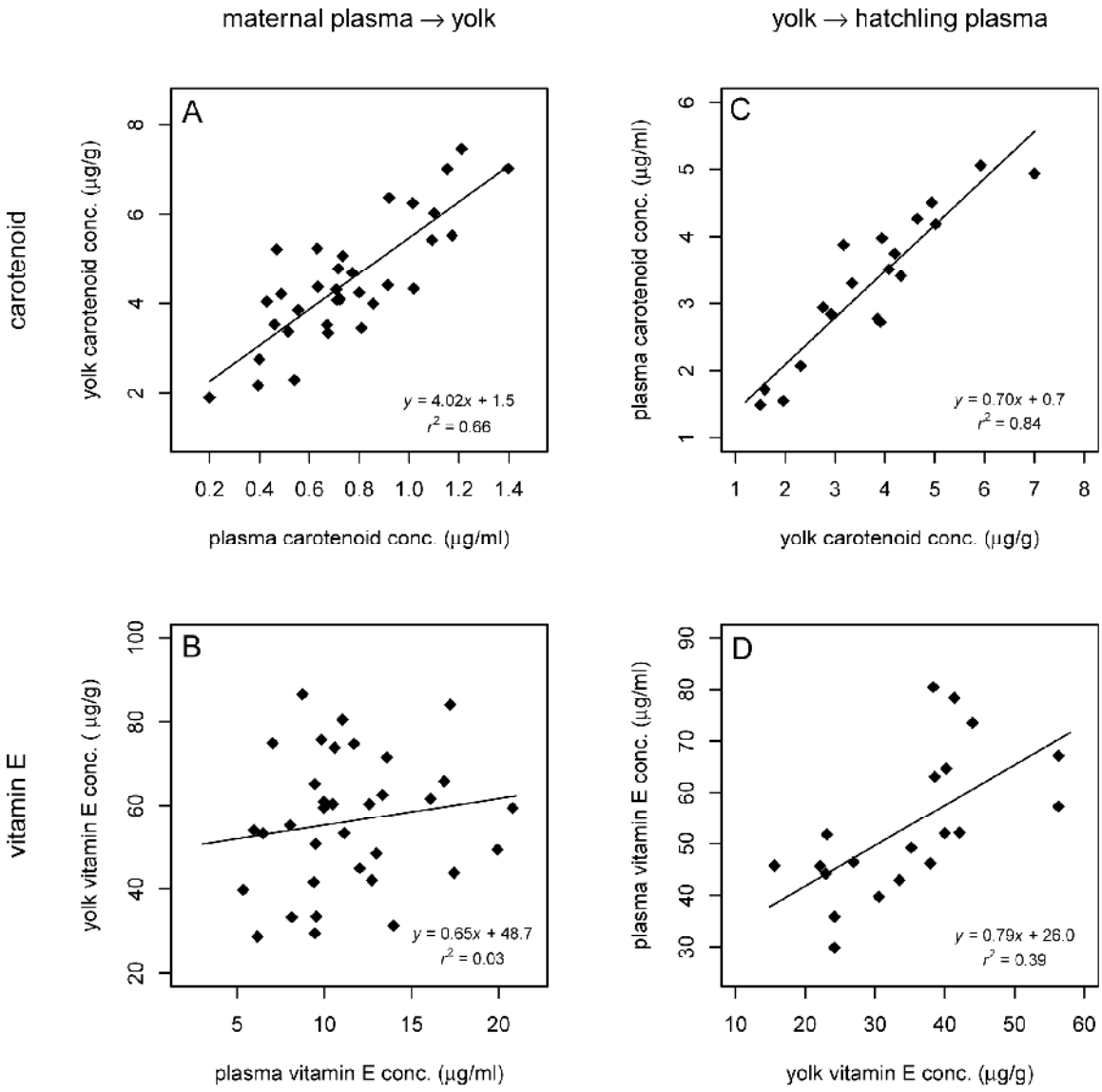



**Figure 4.1.** Relationships between concentrations of (A) carotenoids and (B) vitamin E in maternal blood plasma and egg yolk, and between concentrations of (C) carotenoids and (D) vitamin E in egg yolk and hatchling blood plasma. Lines were fitted using ordinary least squares regression.

mechanisms. Concentrations of vitamin E and carotenoids in egg yolk were not significantly correlated (Pearson's product-moment correlation; $r = 0.08$, $N = 34$, $p = 0.65$); although concentrations were significantly correlated in maternal blood plasma ($r = 0.36$, $N = 34$, $p = 0.036$).

Concentrations of vitamin E and carotenoids in female plasma did not vary according to stage in the nesting season (one-way ANOVA, all $F_{2,33} < 2.0$, $p > 0.15$; see § 4.3.1),

however there was significant seasonal variation in yolk composition. Yolk carotenoid concentrations were higher for females nesting early in the season compared to those nesting at peak or late stages (ANCOVA with maternal plasma levels as a covariate; $F_{2,32} = 4.4$, $p = 0.02$; post-hoc Tukey tests: early vs. peak/late, $p < 0.05$; peak vs. late, $p = 0.93$), while yolk vitamin E concentrations were considerably higher for females laying late in the season ($F_{2,33} = 10.9$, $p < 0.001$; post-hoc tests: late vs. peak/early, $p < 0.005$; early vs. peak, $p = 0.60$). There were no significant interactions between sampling period and maternal plasma levels on concentrations of either vitamin E or carotenoids in yolk (ANCOVA, both $F_{2,30} < 1.1$, $p > 0.35$), indicating that relationships between plasma and yolk antioxidant concentrations did not vary according to stage in the nesting season.

During development, yolk micronutrients are progressively transferred to the developing embryo, so we should expect quantitative relationships between antioxidant levels in yolk, and levels in hatchling tissues. As predicted, for clutches laid in 2007, concentrations of vitamin E and carotenoids in hatchling plasma were both strongly determined by their respective concentrations in egg yolk (linear regression, both $F_{1,19} > 11.0$, $p < 0.003$; **Figures 4.1C & 4.1D**).

Profiles of different carotenoids and tocopherols were similar in egg yolk and maternal and hatchling plasma: lutein and zeaxanthin were consistently the dominant carotenoids

**Table 4.2.** Relative levels of different tocopherols and carotenoids in the blood plasma of nesting green turtles and newly emerged hatchlings.Values expressed as percentages of total vitamin E and carotenoid levels.

| | Maternal plasma | | Hatchling Plasma | |
|---|---|---|---|---|
| | mean ± SE | range | mean ± SE | range |
| | | vitamin E | | |
| α-tocopherol (%) | 97.1 ± 0.3 | 92.8 – 99.7 | 95.9 ± 0.5 | 92.3 – 99.2 |
| γ-tocopherol (%) | 2.9 ± 0.3 | 0.3 – 7.2 | 4.1 ± 0.5 | 0.8 – 7.7 |
| | | carotenoids | | |
| zeaxanthin (%) | 42.1 ± 2.1 | 26.5 – 64.1 | 26.1 ± 2.6 | 10.9 – 56.0 |
| lutein (%) | 40.5 ± 2.4 | 14.7 – 60.4 | 61.1 ± 3.3 | 23.1 – 81.4 |
| unidentified (%) | 17.3 ± 0.5 | 12.5 – 25.1 | 12.8 ± 0.5 | 9.1 – 19.5 |
| | $N = 34$ | | $N = 100$ | |

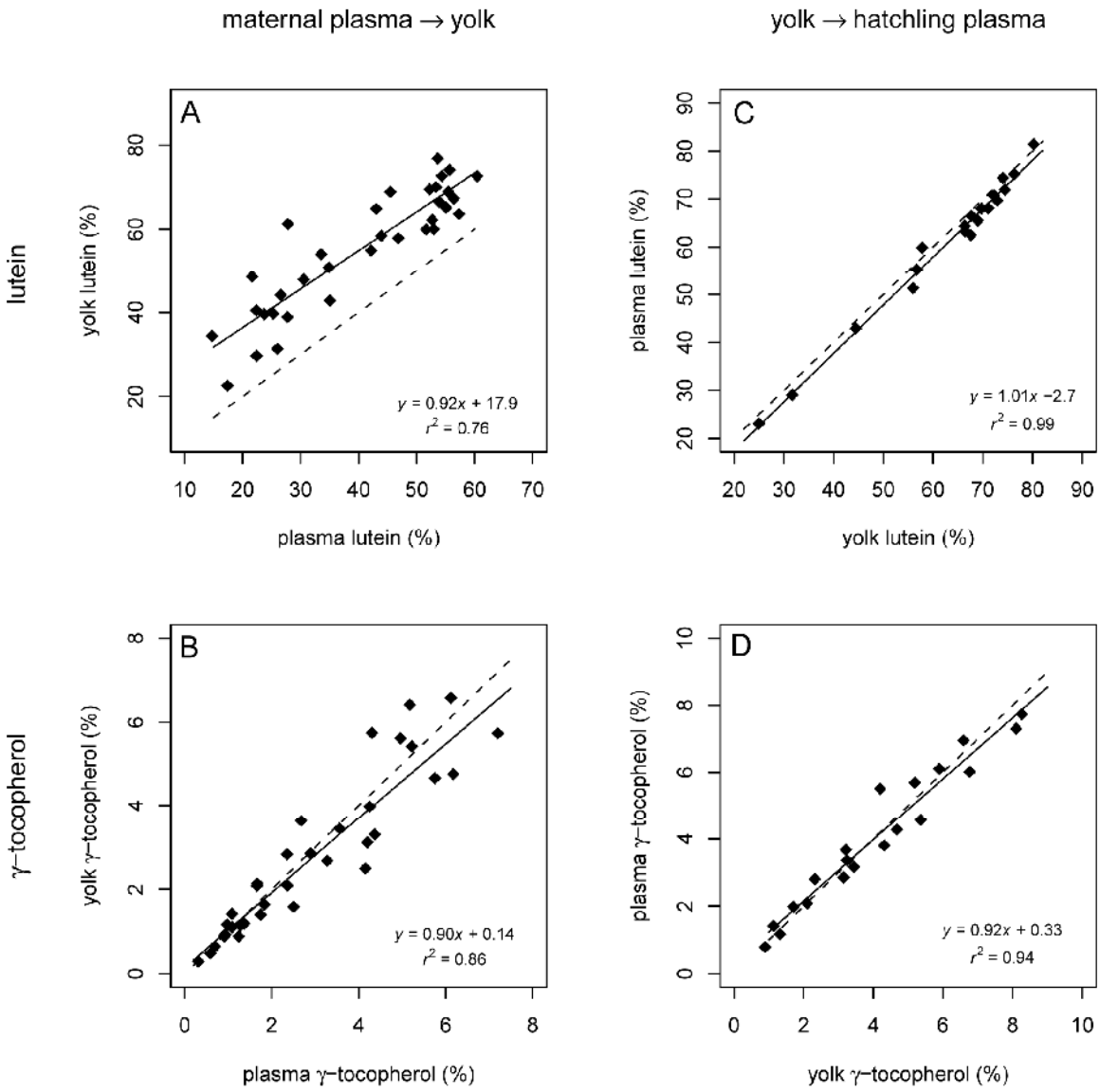



**Figure 4.2.** Relationships between relative levels of (A) lutein and (B) γ-tocopherol in maternal plasma and egg yolk; and between relative levels of (C) lutein and (D) γ-tocopherol in egg yolks and hatchling plasma. Regression lines fit using ordinary least squares regression. Broken lines show the line of equivalence ($y = x$) for comparison.

(with 3 unidentifiable hydroxycarotenoids also isolated) while vitamin E was largely present as the α-tocopherol form, with a smaller proportion of γ-tocopherol accounting for the remainder (**Table 4.2**). However, relative levels of the major carotenoids and tocopherols in eggs varied considerably among clutches, and were strongly predicted by their relative abundance in maternal blood plasma (linear regression all $F_{1,33} > 100$, $p < 0.0001$, $r^2 > 0.75$; **Figures 4.2A & 4.2B**). Proportions of different carotenoids and tocopherols in hatchling plasma also corresponded closely with their relative

abundances in egg yolk ($F_{1,19} > 300$, $p < 0.0001$, $r^2 > 0.94$; **Figures 4.2C & 4.2D**). Thus, variation in maternal carotenoid and vitamin E profiles was quantitatively transferred to eggs and hatchlings.

To assess whether specific carotenoids and tocopherols are selectively transferred to eggs and developing embryos, I used *F*-tests to determine whether the relationship between relative concentrations in blood plasma and egg yolk differed significantly from an equivalent relationship with a slope of 1 and intercept of 0 (i.e. $y = x$). Relationships between relative levels of α- and γ-tocopherol did not differ significantly from equivalence either for transfer from maternal plasma to yolk (*F*-test, $F_{2,32} = 1.6$, $p = 0.21$; **Figure 4.2B**), or from yolk to hatchling plasma ($F_{2,18} = 1.4$, $p = 0.27$; **Figure 4.2D**), suggesting limited discrimination between different tocopherols. Proportions of lutein and zeaxanthin in yolk and hatchling plasma were also close to equivalence suggesting non-specific transfer (*F*-test, $F_{2,18} < 2.5$, $p > 0.10$; **Figure 4.2C**). However, egg yolk contained significantly less zeaxanthin and more lutein than expected from maternal plasma profiles (intercepts significantly different than zero: $F_{1,33} > 24.0$, $p < 0.001$; slopes not significantly different than 1: $F_{1,33} < 2.2$, $p > 0.15$; **Figure 4.2A**), suggesting that lutein may have been preferentially accumulated in eggs.

## 4.5. Discussion

This study has shown that variation in the levels of vitamin E and carotenoids provisioned into eggs by female green turtles strongly influence reserves of these micronutrients in their offspring (**Figures 4.1C & 4.1D**). Relationships between yolk antioxidants and levels in neonates have been demonstrated previously in domestic poultry (e.g. Koutsos et al. 2003; Karadas et al 2005; Lin et al 2005), but have not been found in most studies of wild birds (Biard et al 2005; Biard et al 2007; Saino et al 2008; de Neve et al 2008; but see Ewen et al. 2006), potentially due to the rapid switch to exogenous food sources post-hatching. Thus, the contribution of maternally-derived atioxidants in eggs to neonatal reserves may be quickly replaced by self-feeding or parental rearing in birds (Karadas et al 2005; Biard et al 2007). In contrast, marine turtles have no post-laying parental care, and it is thought that self-feeding does not begin until several days after emergence from the nest, at the culmination of the 'frenzy swimming' phase that carries hatchlings offshore into open-ocean habitats (Salmon &

Wyneken 1987). Consequently, the quantities of vitamin E and carotenoids provided in eggs by mothers may have lasting effects on hatchling reserves and may be an important determinant of antioxidant capacity during an energetically demanding life stage (but see **Chapter 5**).

The factors which regulate maternal deposition of vitamins and carotenoids in eggs are generally poorly understood, although previous studies in turtles suggest that physiological rather than dietary constraints may account for much of the variation among females under natural conditions (**Chapter 3**). At a proximate level, yolk antioxidants may simply vary as a quantitative function of circulating levels in the female. Indeed, concentrations of carotenoids in green turtle eggs were strongly and positively correlated with maternal plasma concentrations (**Figure 4.1A**), as has been reported previously in birds (Bortolotti et al. 2003; Isaakson et al. 2006, 2008). However, I found no such relationship for vitamin E, despite 5-fold variation in maternal plasma concentrations (**Figure 4.1B**). Studies in mammals and birds have shown that plasma concentrations of carotenoids and vitamin E correlate positively with endogenous stores in the liver and adipose tissue (Kardinaal et al. 1995; El Sohemy et al 2002; McGraw and Toomey 2010). Thus, the results of the present study suggest that carotenoid provisioning in eggs is directly proportionate to the size of maternal body reserves, where as vitamin E provisioning is not. Indeed, in zebra finches (*Taeniopygia guttata*), variation in maternal adipose stores was shown to positively predict the concentration of carotenoids deposited in eggs, but was unrelated to vitamin E provisioning (Williamson et al. 2006). The lack of correlation between vitamin E in maternal plasma and yolk could be explained by (at least) three potential mechanisms.

Firstly, it is possible that vitamin E concentrations in eggs are regulated by rates of ovarian uptake from the maternal bloodstream rather than plasma concentrations *per se*, as demonstrated for yolk proteins and lipids in birds (Christians & Williams 2001a, b). However, vitamin E and carotenoids are simultaneously transferred into developing yolk follicles as components of plasma lipoproteins (Surai 2002), so differential rates of uptake cannot explain why yolk carotenoids correlated with maternal plasma concentrations while yolk vitamin E did not. Indeed, the co-transport of vitamin E and carotenoids in plasma lipoproteins probably explain why concentrations of these micronutrients were positively correlated in the plasma of nesting females.

Alternatively, since I measured maternal plasma antioxidant concentrations after yolk formation was complete, it is possible that maternal vitamin E reserves had been

depleted and that this prevented correlations between plasma and yolk levels. Again, this explanation seems unlikely. I previously showed that vitamin E concentrations in eggs increase across successive clutches produced by individual green turtles (**Chapter 3**), which is contrary to the expected trend if maternal vitamin reserves are depleted. Moreover, the same study suggested that maternal carotenoid stores are progressively expended during egg production (**Chapter 3**; see also Bortolotti et al. 2003; Groothuis et al. 2006), yet in the present work plasma carotenoid levels in nesting females remained strongly and positively correlated with yolk levels.

Finally, and most plausibly, the absence of correlations between vitamin E levels in yolk and in the plasma of nesting females could be explained by the dynamic changes in maternal blood biochemistry that accompany yolk formation. The vast majority of yolk constituents are extracted from the maternal bloodstream during a period of intense follicular growth, characterised by transient, oestrogen-dependent increases in circulating levels of many yolk precursors (Ho et al. 1982; Heck et al. 1997; Walzem et al. 1999). Indeed, plasma concentrations of vitamin E increase in response to treatment with exogenous oestrogen in birds (Halifeoglu et al. 2003), and mirror the endogenous production of estradiol during vitellogenesis in alligators (Lance et al. 1983): vitamin levels peak during yolk deposition and then decline to a level similar to males and non-breeding females. I previously suggested that these cyclical changes in plasma oestrogen and vitamin levels during yolk formation could account for the contrasting within-female patterns of vitamin E provisioning in birds and sea turtles (**Chapter 3**). Oestrogen production (and sensitivity to it) also varies dramatically among individual breeding females (reviewed in Williams 2008) and may therefore be a significant determinant of vitamin E concentrations in eggs. Since I measured maternal plasma antioxidants at nesting, when the peak in plasma oestrogen (and therefore potentially plasma vitamin E) has subsided (Rostal et al. 1998), hormonal regulation of vitamin E deposition in eggs would explain the lack of correlation between yolk and maternal plasma concentrations. Experimental approaches using exogenous hormone would be a logical next step in evaluating this mechanism.

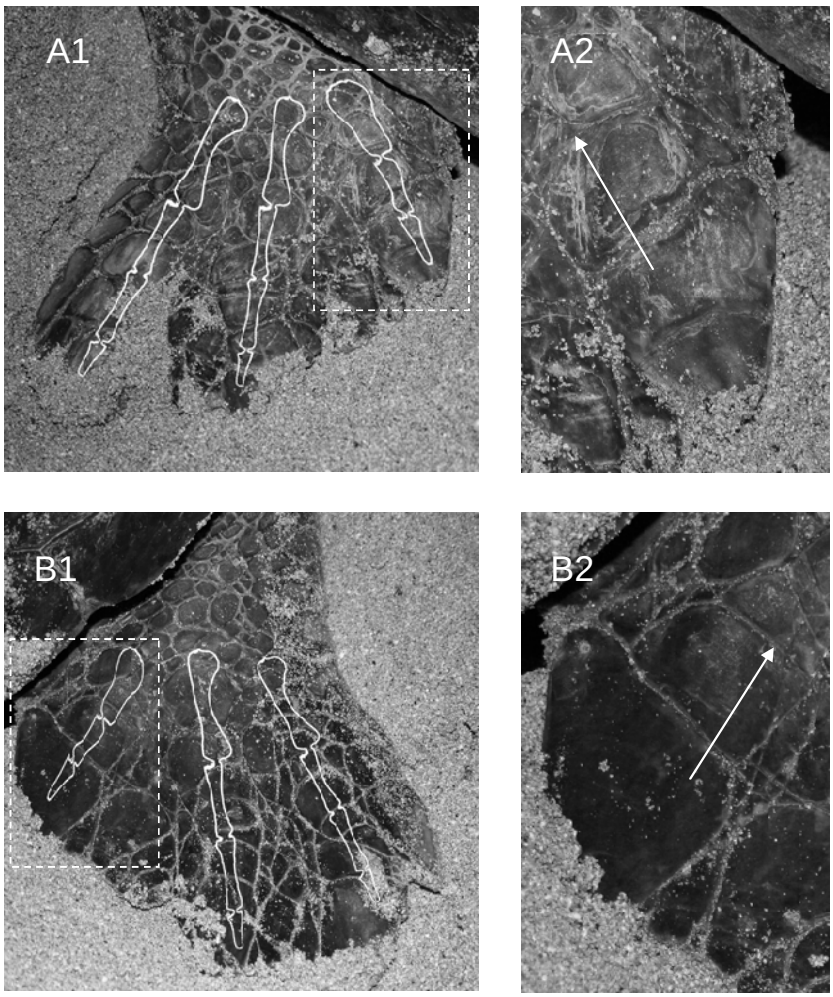
In contrast to absolute concentrations of vitamin E, relative concentrations of α- and γ-tocopherol in egg yolk were strongly predicted by their relative abundance in maternal blood plasma (**Figure 4.2B**), as were relative concentrations of the yolk carotenoids lutein and zeaxanthin (**Figure 4.2A**). Different forms of vitamin E and carotenoids vary in their potency as antioxidants (Edge et al. 1997; Surai 2002) and it has been suggested

that selection may favour the provisioning of specific types or combinations into eggs (Blount et al. 2002b; Royle et al. 2003). I found no evidence for post-absorptive discrimination between α- and γ-tocopherols during transfer to yolk (proportions in maternal plasma and yolk approximated an equivalent relationship; **Figure 4.2B**), however there was a clear preference for maternal transfer of lutein into eggs compared to zeaxanthin: the proportion of lutein in total egg yolk carotenoids was on average 15.0 ± 1.1 % higher than expected from maternal plasma profiles while the proportion of zeaxanthin was 7.1 ± 1.0 % lower (**Figure 4.2A**). Zeaxanthin has more potent free radical quenching properties than lutein (Edge et al. 1997), so this disparity is unlikely to represent an adaptive strategy to enhance the antioxidant status of eggs for offspring (see also **Chapter 5**). A lutein-specific binding protein was recently identified in avian liver which may mediate the selective incorporation of lutein into yolk-targeted lipoproteins (Bhosale & Bernstein 2007); however the functional relevance of this preference for offspring (if any) awaits investigation.

In conclusion, this study has shown that concentrations of fat-soluble antioxidants in the plasma of hatchling green turtles reflect the composition of the egg in which they developed. However, as suggested in **Chapter 3**, the mechanisms which regulate maternal investment in eggs appear to be distinct for carotenoids and vitamin E. Interestingly, carotenoid concentrations in mammalian milk have similarly been shown to correlate with maternal plasma concentrations, whereas levels of vitamin E do not (de Azeredo & Trugo 2008, and references therein), suggesting a more general dichotomy in the mechanisms of antioxidant transfer from mother to offspring. There is currently considerable debate over the extent to which females can adjust egg composition independently of their own physiological state, since this has important implications for adaptive interpretations of egg-mediated maternal effects (Groothuis & Schwabl 2008; Boulinier & Staszewski 2008; **Chapter 8.1**). The results presented here and in **Chapter 3** strongly suggest that carotenoid provisioning in eggs at both intra- and inter-individual levels is constrained by the size of maternal carotenoid reserves; however several lines of evidence point to the active regulation of vitamin E deposition in eggs, potentially involving the action of reproductive hormones. Whether antioxidant transfer to eggs has an adaptive basis ultimately rests in the consequences of maternal provisioning for offspring and maternal fitness and is explored further in **Chapter 5**.

## 4.6 Appendix: Supplementary methods

**Figure A4.3**. The interdigitary vessel (IDV) blood sampling protocol of Wallace and George (2007) as adapted for green turtles. Figures A and B show left and right hind flippers respectively from two different individuals. For clarity, the outlined frames in panels A1 and B1 (broken lines) are magnified in panels A2 and B2 respectively. Blood is most easily sampled adjacent to the medial digit of the hind flipper during the quiescent phase of oviposition (Wallace and George 2007). In green turtles a triplet of large scales overlying the medial digit provide convenient external landmarks for identifying the correct insertion point. After sterilising with an antiseptic spray, the needle is inserted parallel to the digit into the soft skin between the proximal and second of these scales (arrows in panels A2 and B2), using an angle as parallel to the surface of the flipper as is practicable. A 1" 22 gauge needle is sufficient in all cases, and is inserted to a depth of ~ 5 mm before attaching a lithium-heparin coated Vacutainer® tube (Becton-Dickinson). The needle is then slowly advanced until blood enters the tube; although it may be necessary to withdraw the needle slightly to allow blood flow. (NB. Bones of digits are schematic for illustrative purposes only and are not anatomically accurate).



# Chapter 5 Environmentally-induced oxidative stress is not compensated by maternal antioxidant investment in the green turtle.

## 5.1 Abstract

Oxidative stress is an inevitable cost of living and may be an important determinant of fitness, particularly during early life stages. In birds, reptiles and other oviparous animals, females provision their eggs with diet-derived antioxidants such as vitamin E and carotenoids, which is thought to be an adaptive maternal effect to buffer their offspring against the harmful effects of oxidative stress. However, evidence for such a function in wild populations is lacking; particularly since few studies have considered how the offspring developmental environment influences exposure to oxidative stress and the requirements for maternally-derived antioxidants. In this chapter I investigate how the nest site selected by a female and the quantities of fat-soluble antioxidants provided in eggs interact to determine oxidative stress levels in hatchling green turtles (*Chelonia mydas*). Environmental conditions experienced in the nest strongly affected exposure to oxidative stress, as hatchlings from shallower, drier nests had markedly increased blood levels of lipid peroxidation (malondialdehyde; MDA). Oxidative stress was also associated with specific costs, as hatchlings with higher plasma MDA concentrations were smaller and in poorer body condition. However, maternal provisioning of vitamin E and carotenoids in eggs was unrelated to either MDA levels in hatchlings or environmental characteristics of the chosen nest site. Similarly, antioxidant levels in eggs did not affect hatchling survival or body condition, although hatchlings from carotenoid rich eggs were larger, suggesting that maternally derived carotenoids may enhance offspring growth independently of any antioxidant function. This study provides novel evidence that maternal oviposition behaviour can influence offspring exposure to oxidative stress, but challenges the widely held assumption that antioxidant provisioning in eggs is an adaptive maternal effect to compensate for this risk.

## 5.2 Introduction

The need to protect cells and tissues from oxidative damage caused by the reactive by-products of aerobic metabolism is of fundamental importance in all animals (Halliwell & Gutteridge 2007; Monaghan et al 2009). Consequently, a multifaceted antioxidant system comprised of enzymes, metabolites and diet-derived components has evolved to neutralize reactive oxygen species (ROS) and free radicals before oxidative injury can occur (Halliwell & Gutteridge 2007; Catoni et al 2008; Monaghan et al. 2009). An imbalance between the endogenous production of ROS and the capacity of antioxidant systems results in a state of oxidative stress, with widespread damage to lipids, DNA and proteins (Monaghan et al 2009; **Chapter 1**). Oxidative stress may therefore have significant fitness consequences and has recently been highlighted as a potentially unifying mechanism underpinning life-history trade-offs in animals (Constantini 2008; Dowling & Simmons 2009; Monaghan et al 2009).

During early life, a combination of rapid growth and poorly developed endogenous antioxidant defenses may present a high risk of oxidative stress (Surai 2002; Monaghan et al. 2009). In all oviparous vertebrates, females provision their eggs with diet-derived, lipid soluble antioxidants such as vitamin E and carotenoids which are though to provide crucial protection against oxidative stress during early development. For reasons that are not fully understood, the level of antioxidant provisioning in eggs often varies dramatically among females in wild populations (Biard et al. 2005; Saino et al. 2008; Isaakson et al. 2008; **Chapters 3 & 4**) and there has been considerable interest in how such variation might impact on offspring phenotypes (e.g. Biard et al. 2005; Saino et al. 2008; Ewen et al. 2008; **Chapter 1**). However, whilst there is some evidence that yolk levels of vitamin E and carotenoids influence the oxidative stability of yolk lipids and embryonic/neonatal tissues *in vitro* (Surai & Speake 1998; Surai et al. 1999; Blount et al. 2002a, b; McGraw et al. 2005), the assumption that increased provisioning of antioxidants in eggs necessarily translates into improved oxidative stress resistance in offspring has never been tested *in vivo*. This is an important omission given recent suggestions that carotenoids may be weak antioxidants under physiological conditions (Hartley & Kennedy 2004; Constantini & Møller 2008; Isaksson & Andersson 2008; Olsson et al. 2008). Indeed, evidence that egg carotenoid levels influence fitness-related traits in offspring such as survival and body size (often attributed to their putative

antioxidant function) is highly equivocal (reviewed in **Chapter 1**; Biard et al. 2005; McGraw et al. 2005; Svensson et al. 2006; Remes et al. 2007; Saino et al. 2008; Ewen et al. 2008; Grether et al. 2008), and has been little explored with regard to vitamin E provisioning (but see Møller et al. 2008 for a rare exception in radioactively-damaged habitats).

In addition to maternal antioxidant investment, the risk of oxidative stress in offspring may be influenced by a variety of intrinsic and extrinsic factors which modulate the endogenous production of ROS (reviewed in Constantini 2008; Dowling and Simmons 2009; Monaghan et al 2009). For example, recent work has suggested that rapid growth may be a significant determinant of oxidative stress in animals due to increases in metabolic rate and oxygen consumption (Alonso-Alvarez et al. 2007; de Blok & Stoks 2008; Nussey et al. 2009; Hall et al. 2010). Similarly, in many ectothermic species, the rate of lipid peroxidation (oxidative damage to lipids) in tissues increases as a function of ambient temperature (reviewed in Crockett et al. 2008). However, remarkably little is known about how the developmental environment experienced during early life effects exposure to oxidative stress and the requirement for maternally-derived antioxidants.

The interplay between maternal antioxidant provisioning in eggs and the offspring environment generates two sets of predictions, differentiated by the degree of plasticity shown by females (**Figure 5.1**). On one hand, females may be constrained to produce eggs with a certain level of antioxidant protection and/or may select developmental environments independently of antioxidant provisioning. In this instance we might expect offspring from antioxidant rich eggs to perform better in more stressful environments, resulting in egg phenotype × environment interactions for fitness (**Figure 5.1A**; see Rossiter 1998). For example, the effects of maternally-derived antioxidants on oxidative stress levels (and therefore on downstream traits) may be stronger in environments in which offspring grow more rapidly (Hall et al. 2010). Such 'context-dependency' for maternal effects has been well documented with regards to egg size, where the benefits of producing large eggs may only become manifest under conditions of high intraspecific competition, resource scarcity or otherwise adverse environments (Fox & Mousseau 1996; Rossiter 1998; Einum & Flemming 1999; Räsänen et al. 2005). Alternatively, females may tailor the level of antioxidant protection in eggs to a 'perceived' environmental risk of oxidative stress through adaptive adjustments in provisioning (i.e. anticipatory maternal effects; Marshall & Uller 2007) or selection of

nest sites based on a capacity to provide antioxidants (i.e. condition dependent choice; Roosenburg 1996). Such plasticity could obscure relationships between antioxidant provisioning in eggs and offspring phenotypes, but would be evident as correlations between yolk antioxidant levels and potential environmental determinants of oxidative stress (i.e. the stressors eliciting the maternal effect; **Figure 5.1B**).

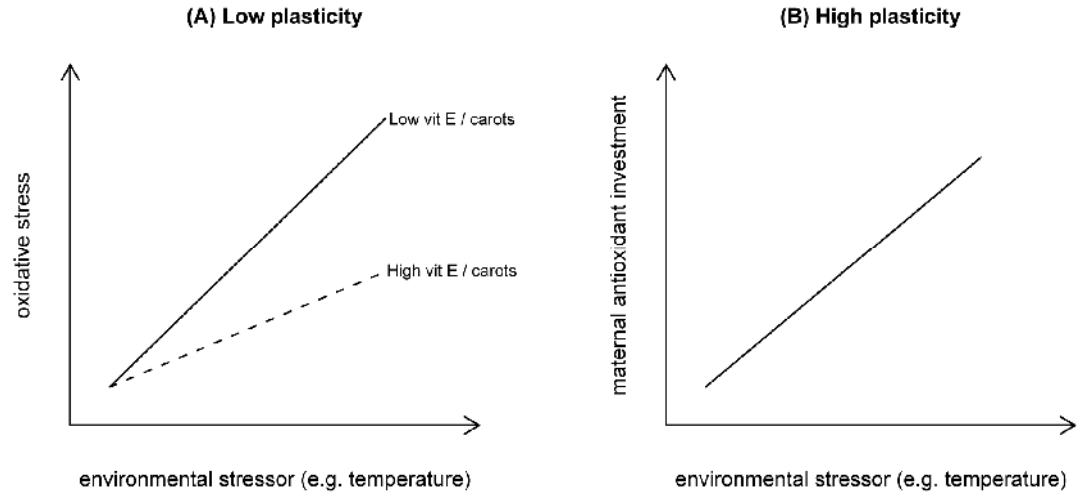



**Figure 5.1.** Outline of hypotheses. (A) If maternal antioxidant provisioning is constrained and/or nest selection is random with respect to the determinants of oxidative stress (i.e. low maternal plasticity), more antioxidant replete eggs might be expected to perform better in more stressful environments; alternatively, (B) if females tailor levels of antioxidant protection in eggs to specific nest sites (i.e. high maternal plasticity), antioxidant investment should correlate with environmental parameters predicted to influence oxidative stress.

In this chapter I investigate how the developmental environment experienced during incubation and maternal antioxidant provisioning in eggs combine to determine oxidative stress levels, hatching success and morphology in hatchling green turtles (*Chelonia mydas*). Previous studies of antioxidant-mediated maternal effects have focused predominantly on birds, where the developmental environment is shaped by a complex integration of parental behaviors and sibling interactions throughout incubation and rearing (all of which may influence antioxidant availability, growth rates and levels of oxidative stress; Saino et al. 2008; Hall et al. 2010). In contrast, sea turtles do not exhibit post-laying parental care, so maternal investment is limited to egg production and nest selection only. Moreover, the microenvironment experienced by eggs buried in

nests on sandy beaches is defined by a small number of measurable abiotic factors, such as temperature and substrate moisture content, which vary among potential nest sites (reviewed in Ackerman 1997). Such factors are known to determine the rate of embryonic metabolism and growth in reptiles (Packard 1991; Birchard 2004), which may in turn influence the risk of oxidative stress and the requirement for antioxidants.

I have previously shown that concentrations of vitamin E and carotenoids in green turtle eggs vary markedly among individual females (**Chapter 3**) and that such variation strongly predicts antioxidant reserves in newly emerged hatchlings (**Chapter 4**). Yolk formation is largely complete prior to arrival at the breeding grounds in marine turtles, so adaptive adjustments of antioxidant provisioning in response to the nest environment seem unlikely (Rostal et al. 1997; Hamann et al. 2003). Nonetheless, a female's capacity to furnish her eggs with antioxidants may influence her selection of nest site (in an evolutionary sense) if environmental cues reliably predict the risk of oxidative stress. I hypothesized that 1) hatchlings developing in environments conducive with rapid growth would experience increased levels of oxidative stress; 2) antioxidant provisioning in eggs would exert stronger effects on oxidative stress levels and hatchling phenotypes under more stressful environmental regimes (**Figure 5.1A**); and/or 3) females that invested less antioxidants in eggs would be constrained to choose less stressful nest environments (**Figure 5.1B**).

## 5.3 Materials & Methods

**5.3.1 Study Site and Sampling Procedures.** The study was conducted at Ascension Island in the South Atlantic Ocean during the 2007 nesting season. Nesting activity at this rookery predominantly runs from January – May, peaking in early March (Godley et al. 2001). Between 17$^{th}$ January and 30$^{th}$ March I selected females ($N$ = 30) located at the early stages of nesting by carrying out nightly patrols of the Long Beach nesting site. Females in this population lay multiple, large clutches of eggs (mean clutch size: 127 ± 5 SE, range: 86 - 165) within a single nesting season. Thus, to avoid subsequent re-sampling all study females were fitted with a unique PIT tag (Passive Integrated Transponder), which was implanted into the triceps muscle of the right fore-flipper following laying to allow identification. Three eggs were sampled from each clutch at approximately the 10$^{th}$, 50$^{th}$ and 10$^{th}$ from last positions in the laying sequence for analysis of antioxidant contents. Eggs were weighed, separated into yolk and albumen

fractions, and aliquots of the homogenized yolk stored at -45 ºC at the study site before transfer on dry ice to -80 ºC storage at our laboratory in the UK.

Nest locations were marked and fitted with a cage from day 45 of incubation to trap emergent hatchlings. Hatching and emergence from the nest is separated by a variable period of 4 – 7 days in sea turtles ('emergence lag'; summarized in Godfrey and Mrosovsky 1997), however it is possible to reliably estimate development times (period of embryonic development to hatching) and emergence lags from temperature profiles in the nest (see Appendix § 5.6). Within 24 hours of emergence, all hatchlings were weighed (± 0.1 g) and straight carapace length (SCL) measured using digital calipers (± 0.1 mm). For a randomly selected sample of hatchlings from each clutch (*N* = 10 - 12*)* we collected ca. 50 µl blood from the dorsal cervical sinus using a 8mm × 30G needle (BD Micro-Fine, Becton-Dickinson, NJ, USA) for analysis of oxidative stress (see below). Blood was fractionated by centrifugation at 10,000 × g for 5 minutes and the plasma layer recovered and stored as described for yolk samples. Following hatchling emergence, nests were excavated to confirm clutch size and hatching success from the number of hatched and unhatched eggs.

**5.3.2 Nest Environment.** Marine turtles bury their eggs in nest chambers excavated on sandy beaches. For each nest we recorded the sand moisture content, the incubation temperature and the depth of the chamber below the surface. The volumetric water content of sand in the nest chamber walls was measured in triplicate at the time of laying (± 1%) using an impedance soil moisture probe (ThetaProbe ML2x, Delta-T Devices, Cambridge UK) connected to an HH2 moisture meter (Delta-T Devices). The probe was calibrated to Long Beach sand characteristics as per the manufacturer's instructions, and the output is known to be stable across the range of salinities encountered in beach sand (Miller & Gaskin 1999). Incubation temperature was measured using archival temperature loggers (± 0.3 ºC; Tinytag, Gemini Data Loggers, Chichester, UK) placed in the centre of each clutch, following sampling of the 50$^{th}$ egg, and programmed to record at 4-h intervals. Loggers were subsequently retrieved during nest excavation. Nest depth relative to the surface was recorded post-emergence during the nest excavation phase. Prior to excavation a surface line was secured across the sand and nest depth was then measured as the vertical distance from the surface marker to the top of the clutch mass along a weighted plumb line. Vertical distance to the bottom of the egg chamber could not be measured accurately in all cases as nest chambers collapsed during removal of hatched eggs for clutch size analysis. However depth to the

bottom of the nest chamber was highly correlated with depth to the top of the clutch mass for those nests where measurements could be made (Pearson's correlation, $r$ = 0.93, $p < 0.0001$, d.f. = 25).

**5.3.3 Quantifying Oxidative Stress.** Egg yolk is rich in unsaturated lipid and therefore embryos are potentially vulnerable to lipid peroxidation during development (Surai et al. 1999). We assessed plasma concentrations of malondialdehyde (MDA), a secondary breakdown product of lipid peroxidation and therefore an ultimate measure of oxidative stress, relevant to the functional consequences of carotenoid and vitamin E provisioning into eggs. Plasma MDA levels were assayed by HPLC following derivatization with thiobarbituric acid (TBA), as described by Agarwal & Chase (2002). Briefly, a 15 µL aliquot of plasma was heated at 100 ºC for 1 h in the presence of 120 µL of phosphoric acid solution (0.44 M) and 30 µL of aqueous TBA solution (42 mM), plus 15 µL of butylated hydroxytoluene (0.05% w/v in 95% ethanol) as an antioxidant. After cooling on ice, 75 µL of *n*-butanol was added and samples were vortex mixed (20 s) and centrifuged for 3 minutes at 13,000 × g and 4ºC to separate the phases. The upper organic phase containing the MDA-TBA adduct was recovered and 20 µl injected onto a Dionex HPLC system (Dionex Corporation, CA, USA) fitted with a Hypersil 5 µ ODS 100 × 4.6 mm column (Hewlett-Packard). Separation used a mobile phase of methanol-buffer (40:60, v/v) - the buffer being 50 mM aqueous potassium monobasic phosphate (adjusted to pH 6.8 with 5 M potassium hydroxide) – running isocratically for 3.5 min at a flow rate of 1 mL $min^{-1}$. Data were collected by fluorescence detection (Dionex RF2000) at wavelengths of 515 nm (excitation) and 553 nm (emission). Plasma MDA levels were calibrated using parallel assay standards of 1,1,3,3-tetraethoxypropane (TEP) serially diluted in 40% ethanol solution.

**5.3.4 Yolk antioxidants.** Levels of carotenoids and vitamin E in eggs were quantified using high performance liquid chromatography as described in **Chapters 2 and 3**, respectively.

**5.3.5 Statistical analyses.** Incubation temperature of sea turtle clutches is a function of sand temperature but also the metabolic heat produced by embryos, which is proportional to clutch size and increases in the latter stages of development (Broderick et al. 2001; Appendix **Figure A5.1**). Thus, nest temperature increases over the incubation period following a logistic curve with lower and upper asymptotes (Appendix **Figure A5.1**). For the analysis of maternal antioxidant provisioning in

relation to environmental variables, we used lower asymptotic temperature to describe the chosen nest environment, as it was independent of metabolic heating and reflects sand temperature at the time of nest selection and laying (see Appendix § 5.6.1). Analyses of effects on hatchling phenotypes used mean incubation temperature, as it best describes the overall thermal environment experienced by developing embryos, and was largely determined by laying date due to seasonal climatic changes at Ascension Is. (see Appendix § 5.6.1); clutch size was included as a covariate in all models to control for metabolic effects on the nest environment.

Variables used to describe the nest environment (nest depth, mean incubation temperature, volumetric sand water content) were not significantly correlated (Pearson's product-moment correlation, all pairwise correlations $p > 0.10$). Due to seasonal climatic gradients, laying date was significantly related to both mean incubation temperature (linear regression $F_{1,29} = 30.4$, $r^2 = 0.52$, $p < 0.001$) and sand moisture ($F_{1,28} = 6.5$, $r^2 = 0.20$, $p = 0.016$), and therefore was not included in models with environmental variables to avoid colinearity of predictors. Antioxidant provisioning in eggs did not vary significantly with laying date (linear regression, $F_{1,28} < 0.35$, $p > 0.5$).

Of the 30 clutches we monitored, sand moisture could not be measured for a single clutch as the position of the female during laying prevented access. All nests produced hatchlings, and plasma was sampled from 10-12 hatchlings selected at random from each clutch (although sufficient blood plasma for oxidative damage assays could not be obtained for 2 clutches due to constraints on time during the peak laying period) giving 284 hatchlings from 27 clutches for plasma lipid peroxidation assays. Levels of vitamin E and carotenoids in eggs were determined as the mean yolk concentrations of 3 eggs from the start, middle and end of each clutch. Vitamin E and carotenoid content of eggs did not vary according to position in the laying sequence (GLMM, all $\chi^2_2 < 1.3$, $p > 0.50$), and within-clutch variation was negligible compared to variation among clutches (within-subject variance component < 2 %), so clutch means were used for statistical analysis.

Plasma MDA levels and hatchling morphometrics were analyzed using general linear mixed models (GLMM) with clutch identity included as a random factor. Hatching success was analyzed as a binomial variable (number hatched / number unhatched) in generalized linear models (GLM) with a quasibinomial error structure to account for overdispersion (Crawley 2007). To avoid overparameterization, the number of

parameters to be estimated in all models, including interaction terms, was limited to *n*/3 (Crawley 2007). Thus, the main effects of nest environmental parameters (depth, mean incubation temperature, volumetric sand water content), clutch size and yolk levels of vitamin E and carotenoids were initially included in models, and 2-way interactions tested individually against the full model. Hatchling body condition was evaluated in models with body mass as the response variable and hatchling straight carapace length (SCL) included as a covariate. Egg mass was also included as a covariate in analyses of hatchling morphology. Minimal adequate models were obtained by stepwise deletion of nonsignificant predictors from maximum likelihood models, starting with interaction terms ($\alpha = 0.05$). Significance levels of deleted terms were assessed using likelihood ratio tests of full versus reduced models for GLMM (which fit a $\chi^2$ distribution), and using type III *F*-tests for quasibinomial GLMs. Fitted models were checked for normality of errors and homoscedasticity. All analyses were carried out using the R 2.9.2 statistical package (R Development Core Team 2009).

## 5.4 Results

The development rate of green turtle hatchlings (days to hatching) was largely predicted by nest temperature, with an additional effect of nest depth: hatchlings developed more rapidly in nests that were hotter and shallower (multiple regression; **Table 5.1** and Appendix Figure A5.2). Moreover, the temperature-dependence of development rate was close to theoretical predictions from metabolic models (see Appendix), suggesting that nest temperature strongly influenced embryonic metabolism. However, neither nest temperature nor development rate significantly affected the level of oxidative damage in hatchlings. Plasma concentrations of malondialdehyde (MDA), a biomarker of lipid

**Table 5.1.** Variation in the development times of green turtle clutches in relation to nest environment parameters (nest depth, mean incubation temperature, sand volumetric water content). Results are from multiple regression (multiple $r^2 = 0.84$).

| Variable | *F* | d.f. | *P* | Estimate (SE) |
|---|---|---|---|---|
| Sand moisture | 0.42 | 1,26 | 0.52 | 0.60 (0.21) |
| Nest depth | 4.8 | 1,27 | 0.037 * | 0.04 (0.02) |
| Mean temperature | 104.2 | 1,28 | < 0.001 *** | -3.92 (0.38) |

peroxidation, varied considerably amongst clutches (mean = 5.1 ± 0.2 µM, range = 3.4 – 6.4 µM), but were not predicted by development rate (GLMM, $\chi_1^2$ = 1.1, $p$ = 0.30), mean nest temperature (**Table 5.2**), or any other nest temperature indices (asymptotic maximum, rate of increase [see Appendix § 5.6.1]; GLMM, all $\chi_1^2$ < 0.15, $p$ > 0.70).

However, independent of its effects on developmental rate, the nest environment strongly influenced the risk of oxidative stress: hatchlings from shallower nests had significantly higher plasma concentrations of MDA, particularly when the sand moisture content was low (depth × moisture interaction; **Table 5.2** & **Figure 5.2**). Nest depth was not significantly related to female size, measured in terms of carapace length (linear regression $F_{1,28}$ = 1.9, $p$ = 0.17; estimate = -0.81 ± 0.58), suggesting that smaller

**Table 5.2.** Variation in hatchling plasma malondialdehyde (MDA) concentrations, body size and hatching success in wild green turtle clutches in relation to the nest environment (nest depth, mean incubation temperature, sand moisture) and egg antioxidant levels (vitamin E, carotenoids). Main effects and significant interactions are presented.

| Variables | $\chi_1^2$ | $P$ | Estimate (SE) |
|---|---|---|---|
| Plasma MDA concentration (GLMM) | | | |
| Nest depth | 14.5 | < 0.001 *** | -0.036 (0.008) |
| Sand moisture | 2.59 | 0.10 | -0.141 (0.09) |
| Depth × moisture | 5.75 | 0.016 * | -0.013 (0.005) |
| Yolk carotenoids | 0.53 | 0.47 | 0.081 (0.11) |
| Mean temperature | 0.12 | 0.73 | -0.059 (0.17) |
| Clutch size | 0.08 | 0.78 | 0.001 (0.005) |
| Yolk vitamin E | 0.03 | 0.86 | -0.002 (0.01) |
| Hatchling carapace length (GLMM) | | | |
| Plasma MDA | 12.4 | < 0.001 *** | -0.219 (0.06) |
| Yolk carotenoids | 5.32 | 0.02 * | 0.403 (0.17) |
| Sand moisture | 1.18 | 0.28 | -0.129 (0.12) |
| Mean temperature | 1.32 | 0.25 | 0.282 (0.25) |
| Nest depth | 1.70 | 0.19 | 0.015 (0.01) |
| Clutch size | 0.34 | 0.56 | 0.003 (0.007) |
| Yolk vitamin E | 0.37 | 0.55 | -0.008 (0.01) |
| Hatching success (quasibinomial GLM) | | | |
| | $F_{d.f.}$ | $P$ | |
| Clutch size | $2.49_{1,26}$ | 0.13 | 0.012 (0.008) |
| Mean temperature | $1.59_{1,25}$ | 0.22 | 0.357 (0.286) |
| Yolk carotenoids | $0.71_{1,24}$ | 0.41 | 0.164 (0.19) |
| Plasma MDA | $1.34_{1,23}$ | 0.26 | -0.285 (0.25) |
| Yolk vitamin E | $0.37_{1,22}$ | 0.55 | 0.011 (0.02) |
| Nest depth | $0.22_{1,21}$ | 0.64 | 0.008 (0.02) |
| Sand moisture | $0.006_{1,20}$ | 0.94 | 0.013 (0.16) |

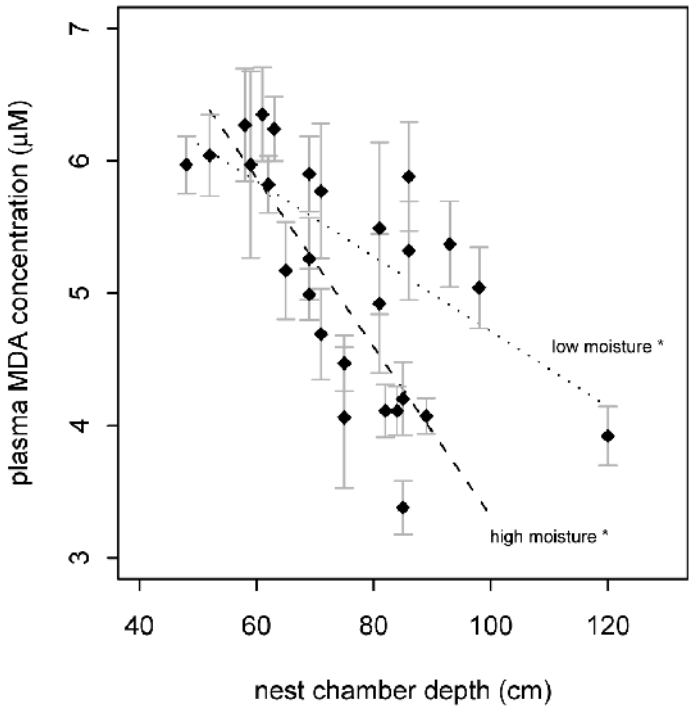
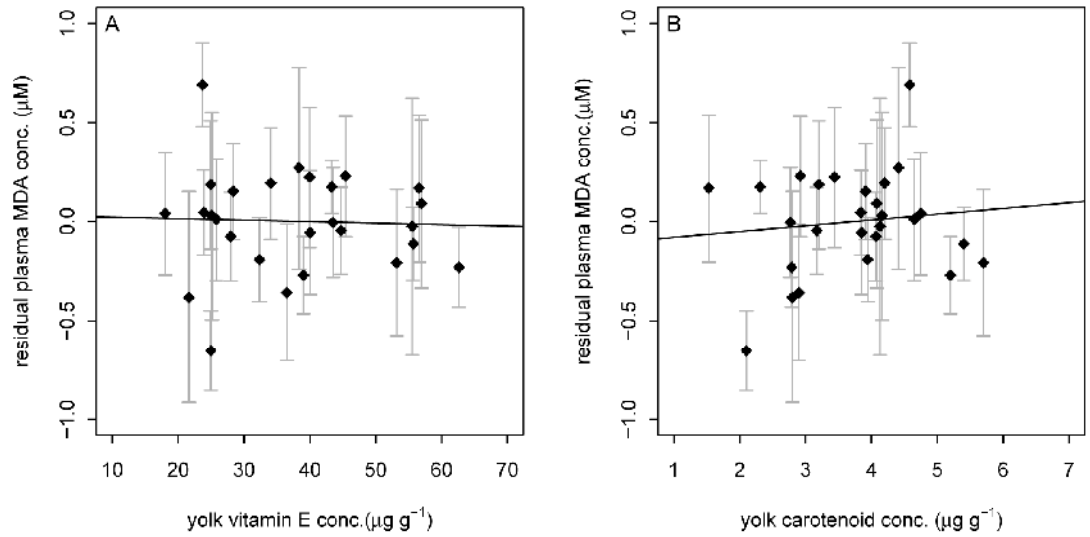



**Figure 5.2.** Interactive effect of nest depth and sand volumetric water content on plasma malondialdehyde (MDA) concentrations in hatchling green turtles. Clutch means ± SE are shown ($N$ = 27). Regression lines for different levels of the moderator (sand moisture) were added using the 'simple slopes' approach (Preacher et al 2006), taking parameter estimates from the minimal adequate model and conditional values for sand moisture of mean ± 1 SD*.

females were not constrained to dig shallower nests. Nest depth and sand moisture content also affected the interval separating hatching and emergence of hatchlings from the nest ('emergence lag'; see Appendix § 5.6.2), although emergence lag was not a significant predictor of plasma MDA levels when nest environmental parameters were included in the model (GLMM, $\chi_1^2$ = 1.1, $p$ = 0.30). Thus, nest depth and sand moisture modulated the risk of oxidative stress in hatchlings independent of effects on development rate or post-hatching emergence lag. Hatchlings with higher plasma concentrations of MDA were smaller (**Table 5.2**) and in poorer body condition (body mass controlling for carapace length; GLMM, $\chi_1^2$ = 32.4, $p$ < 0.001), suggesting that oxidative stress had deleterious effects on growth, although clutch mean concentrations of MDA in hatchling plasma were unrelated to hatching success (**Table 5.2**).

Given that the nest environment modulates the risk of oxidative stress in hatchlings, maternal antioxidant provisioning in eggs may be expected to exert stronger effects on hatchling phenotypes in certain environments (i.e. antioxidant × environment

**Figure 5.3.** Relationships between concentrations of (A) vitamin E and (B) carotenoids in green turtle eggs, and residual hatchling plasma malondialdehyde (MDA) levels from the minimum adequate model (Table 5.2). Clutch means ± SE are shown (*N* = 27).




interactions; **Figure 5.1A**) and/or females may select nest sites based on their capacity to provide adequate antioxidant protection (i.e. antioxidant-environment correlations; **Figure 5.1B**). Yolk antioxidant concentrations varied considerably among clutches (vitamin E: mean = 37.4 ± 12.7 SE μg/g, range = 18.0 – 62.6 μg/g; carotenoids: 3.7 ± 0.2 μg/g, range = 1.5 – 5.7 μg/g), although concentrations of vitamin E and carotenoids were not significantly correlated (Pearson's $r$ = 0.10, d.f. = 28, $p$ = 0.58). However, vitamin E and carotenoid deposition in eggs was not significantly correlated with any nest environment parameter (**Table 5.3**), and did not influence plasma MDA concentrations in hatchlings, either as main effects (**Table 5.2** and **Figure 5.3**) or in

**Table 5.3.** Variation in maternal antioxidant provisioning in eggs in relation to clutch size and environmental characteristics of the nest site (nest depth, sand temperature, sand moisture) from multiple regression models.

| | Vitamin E | | | Carotenoids | | |
|---|---|---|---|---|---|---|
| Variable | $F_{d.f.}$ | $P$ | Estimate (SE) | $F_{d.f.}$ | $P$ | Estimate (SE) |
| Sand moisture | 0.21 $_{1,26}$ | 0.65 | 0.74 (1.60) | 0.04 $_{1,25}$ | 0.84 | -0.030 (0.15) |
| Sand temperature | 0.06 $_{1,25}$ | 0.81 | 0.97 (3.89) | 0.58 $_{1,27}$ | 0.45 | -0.235 (0.31) |
| Nest depth | 0.41 $_{1,27}$ | 0.53 | 0.10 (0.16) | 1.96 $_{1,28}$ | 0.17 | -0.018 (0.01) |
| Clutch size | 4.74 $_{1,28}$ | 0.038* | 0.20 (0.09) | 0.10 $_{1,26}$ | 0.75 | 0.003 (0.01) |

interaction with nest depth, sand moisture or mean nest temperature (GLMM, all two-way interactions, $\chi^2_1 < 1.3$, $p > 0.25$).

In addition to effects on oxidative stress, I also examined how antioxidant provisioning and environmental variables affected clutch survival and hatchling size and condition, since previous studies have linked maternal antioxidant provisioning with offspring viability and morphology (e.g. Biard et al. 2005; Berthouly et al. 2008a; Saino et al. 2008). Hatching success was highly variable among clutches (mean = 86.5 ± 2 SE %, range = 51 – 99 %), but was not significantly related to environmental variables, yolk antioxidant concentrations (**Table 5.2**), or any interactions between them (binomial GLM, all two-way interactions $F_{1,19} < 1.0$, $p > 0.30$). Similarly, hatchling body condition was not predicted by antioxidant levels in eggs (both $\chi^2_1 < 0.1$, $p > 0.80$), although was reduced in hatchlings from deeper nests ( $\chi^2_1 = 4.38$, $p = 0.036$; all other environmental variables and environment × antioxidant interactions, $p > 0.1$). Interestingly, however, clutches with higher carotenoid concentrations gave rise to larger hatchlings (longer carapace length; **Table 5.2** and **Figure 5.4**). This is unlikely to be an antioxidant-mediated effect as hatchling size was not significantly related to egg concentrations of vitamin E (**Table 5.2**), and was not influenced by interactions between concentrations of antioxidants in yolk and environmental variables or oxidative damage in hatchling plasma (GLMM, all two-way interactions $\chi^2_1 < 1.0$, $p > 0.30$), suggesting that yolk carotenoids may influence hatchling size through non-antioxidant pathways.

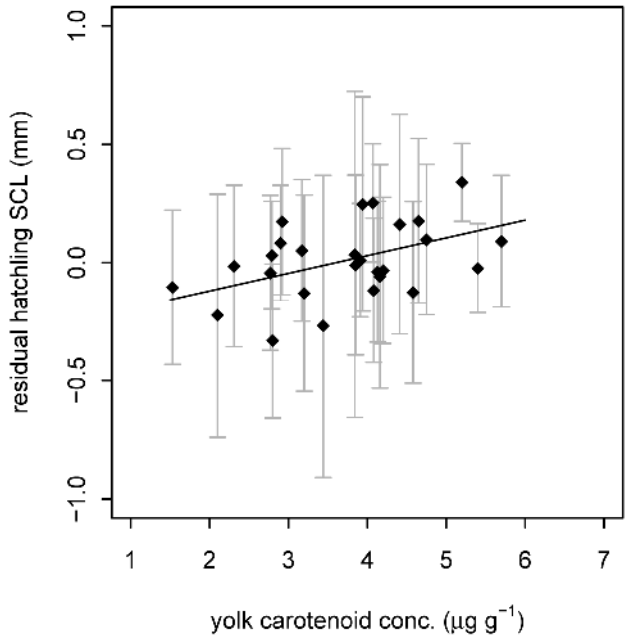



**Figure 5.4.** Relationship between carotenoid concentration in eggs and residual hatchling carapace length (SCL) after controlling for plasma levels of oxidative damage. Clutch means ± SE are shown ($n$ = 27).

## 6.5 Discussion

Females may influence the phenotypes of their offspring via several non-genetic pathways, including the resources and the developmental environment they provide; however, whether such maternal effects necessarily have an adaptive basis has been questioned (reviewed in Heath & Blouw 1998; Marshall & Uller 2007). This study presents novel evidence that the nest site selected by a female can unnecessarily expose her offspring to deleterious oxidative stress. However, we found no evidence that the provisioning of vitamin E and carotenoids in eggs is an adaptive maternal effect to compensate for this risk.

Contrary to our original hypotheses, developmental rate was not a significant determinant of oxidative stress levels in green turtle hatchlings. As in other reptilian species, the rate of development was largely determined by the influence of nest temperature on embryonic metabolism (reviewed in Birchard 2004; see Appendix § 5.6.2); however neither developmental rate nor nest temperature significantly influenced plasma levels of lipid peroxidation (malondialdehyde; MDA) in hatchlings. This contradicts recent evidence that oxidative stress is associated with rapid growth in animals (Alonso-Alvarez et al. 2007; de Blok & Stoks 2008; Nussey et al. 2009; Hall et al. 2010); although these previous studies focused on growth in juveniles rather than embryonic development, and in some cases did not include ultimate measures of oxidative damage (Alonso-Alvarez et al. 2007; de Blok & Stoks 2008). Offspring sex is also temperature-dependent in marine turtles, so the lack of relationship between nest temperature and plasma MDA levels indicates that the variation in oxidative stress which we observed was not sex-biased.

The risk of oxidative stress in hatchlings was largely determined by the depth and sand moisture content of the nest chamber, as we found significantly elevated plasma MDA concentrations in hatchlings from shallower, drier nests (**Figure 5.2**). This effect may be related to the respiratory environment experienced by embryos and neonates. During development, sea turtle embryos consume oxygen in the air space surrounding the eggs thereby lowering oxygen tensions in the nest (Ackerman 1977; Wallace et al. 2004; Miller 2008). Since gas exchange occurs primarily through diffusion with the surface (Ackerman 1977), deeper nests with wetter sand (reduced air-filled pore space) have increased restrictions to gas flux and develop more oxygen-depleted atmospheres

(Ackerman 1981, 1997; but see Miller 2008). Low atmospheric oxygen tensions are associated with reduced oxidative damage to embryonic tissues *in vitro* (Burton 2003; Kitagawa et al 2004), and can depress metabolic rate and blood oxygen saturation *in ovo* (Kam 1993; Giussani et al 2007), which may account for the reduced oxidative stress in deep, wet nests. Low oxygen environments also typically extend developmental times in reptiles (Ackerman 1981; Warbuton et al 1995; Miller 2008) which may account for the slower development of hatchlings in deeper nests. However, the effects of nest depth on oxidative stress were largely independent of development rate, suggesting environmental influences may have operated late in development or post-hatching. Hatching itself is likely to represent a significant oxidative challenge in reptiles, as in birds, with the switch from chorioallantoic to pulmonary respiration and sudden exposure to atmospheric oxygen tensions (Gaal et al. 1995; Blount et al. 2000; Surai 2002), and it is possible that low oxygen environments in deeper, wetter nests buffered hatchlings from oxidative damage during this transitional phase.

Oxidative stress was associated with specific costs, as hatchlings with higher plasma levels of lipid peroxidation were smaller and in poorer body condition. Interestingly, this suggests causality in the opposite direction to that reported previously, where oxidative stress has been observed as a consequence of rapid growth (Nussey et al. 2009; Hall et al. 2010) rather than an apparent determinant of growth capacity. One potential explanation is that free radical damage to yolk-derived lipids restricted embryonic growth. Yolk is rich in polyunsaturated fatty acids, which provide fuel for embryonic metabolism, but are also vulnerable to damage caused by ROS (Surai et al. 1999; Blount et al. 2002a, b; McGraw et al. 2005). Although it is generally expected that oxidative stress has deleterious consequences for fitness, there is scant empirical evidence of such costs in wild animals (Constantini 2008; Dowling & Simmons 2009; Monaghan et al 2009). Reduced body size and condition at hatching may be potentially costly given that previous studies of turtles have suggested that smaller hatchlings suffer increased predation in early life (Gyuris 2000; Janzen et al. 2000; but see Congdon et al. 1999).

Taken together, these results suggest that maternal oviposition behaviour can expose offspring to oxidative stress which in turn may have deleterious consequences for growth. In all oviparous vertebrates, females provision their eggs with fat-soluble antioxidants, which is hypothesised to be an adaptive maternal effect to buffer offspring against the harmful effects of oxidative stress (Blount et al. 2000; Surai 2002; McGraw

et al. 2005; Biard et al. 2005). However, despite marked variation in vitamin E and carotenoid concentrations among clutches (3-fold and 4-fold respectively), there were no relationships between levels of yolk antioxidants and lipid peroxidation in hatchlings (**Figure 5.3**), hatchling body condition or hatching success. Given that the offspring environment was an important determinant of oxidative stress, the relationship between yolk antioxidants and oxidative stress may not simply be additive (**Figure 5.1A**); for example, the benefits of maternally-derived antioxidants may only become apparent in stressful environments when high rates of free radical production exceed the capacity of endogenous antioxidant systems (Surai 2002). However, plasma levels of lipid peroxidation in hatchlings were not predicted by interactions between nest depth, temperature, moisture or development times and antioxidant levels in eggs, indicating that maternal antioxidant provisioning was not a significant determinant of oxidative stress resistance across the range of natural nest environments. We also found no significant correlations between nest conditions and maternal antioxidant provisioning suggesting that females did not adjust nest site selection based on their capacity to provide antioxidant protection (**Figure 5.1B**).

The results of this study are consistent with recent evidence that carotenoids are minor antioxidants for animals *in vivo* (Hartley & Kennedy 2004; Constantini & Møller 2008; Isaksson & Andersson 2008; Olsson et al. 2008) which may account for the weak or equivocal effects of yolk carotenoids on oxidative stress and/or survival of hatchlings reported for many species (this study, Biard et al. 2005; Svensson et al. 2006; Remes et al. 2007; Ewen et al. 2008; but see Surai & Speake 1998 and McGraw et al. 2005; **Chapter 1**). In birds, provisioning of eggs and neonates with carotenoids has been shown to effect morphology (Biard et al. 2005; Berthouly et al. 2008a; Saino et al. 2008; Hall et al. 2010), which has often been attributed to antioxidant protection afforded during growth. We also found a significant positive relationship between yolk carotenoid levels and hatchling body size (**Figure 5.4**), however hatchling size was not significantly related to yolk levels of vitamin E, or interactions between yolk levels of antioxidants and plasma MDA, as would be expected if the effect of carotenoids on hatchling size was mediated by antioxidant activity. Rather, our results suggest that non-antioxidant properties of carotenoids may account for their effects on body size (see also Biard et al. 2005 and Saino et al. 2008). Carotenoids are known to influence the regulation of cell signaling and growth factor expression (Stahl et al. 2002; Sharoni et al. 2004), which may affect embryo and neonate development.

In direct contrast to carotenoids, the importance of vitamin E as a chain-breaking antioxidant in lipid peroxidation reactions is undisputed (reviewed in Halliwell and Gutteridge 2007). Nonetheless, despite 3-fold variation in levels of vitamin E among clutches, this was not significantly associated with levels of MDA in hatchling plasma. One possible explanation is that females were unable to provision their eggs with sufficient vitamin E to control oxidative stress to any great extent. A certain level of antioxidant protection from vitamin E is necessary to enable embryonic development to proceed (Surai 2002; Debier & Larondelle 2005), and the lack of relationships between vitamin E provisioning and hatching success suggests that females generally met this requirement. However, absolute protection against lipid peroxidation may be impossible other than at exceptionally high doses of vitamin E, perhaps beyond the physiologically normal range (e.g. Roberts et al. 2007). Alternatively, studies in reptiles and fish have suggested that the capacity of vitamin E to suppress lipid peroxidation plateaus above a relatively low dietary intake (Huang et al. 2003; Huang & Lin 2004). Increases in vitamin E provisioning above this threshold may not, therefore, reduce levels of oxidative stress in hatchlings. We cannot distinguish between these possibilities because the dose-response relationship for yolk vitamin E and offspring susceptibility to oxidative stress has not been established for this (or any) species. In any case the lack of significant relationships between yolk vitamin E provisioning, hatching success and oxidative stress in surviving hatchlings, indicates that any antioxidant effects of vitamin E are not a key determinant of early life oxidative stress or survival in this species.

The adaptive value of maternal effects must ultimately depend on the predictability of their outcomes for *maternal* fitness (Marshall & Uller 2007). In turtles, selection may favour mothers that provide a threshold level of antioxidant protection and nest conditions which permit successful embryonic development. In contrast, high extrinsic mortality risk in hatchlings means that survival to maturity is likely to be highly stochastic and beyond maternal influence (survival estimates range from 0.01 – 0.1 %; Frazer 1986). Thus, compensatory provisioning of antioxidants to suppress sub-lethal levels of oxidative stress in hatchlings may not predictably enhance maternal fitness; similar uncertainty may explain why females do not consistently construct deep nests which confer reduced oxidative stress risks to hatchlings. Our results underline the importance of interpreting the potential adaptive basis of maternal effects within the context of life histories (Marshall & Uller 2007). In taxa which have a high extrinsic mortality risk in early life, such as turtles and fish, the optimal maternal strategy may be

to produce large numbers of young with investment sufficient only to allow embryogenesis and successful hatching. However, in other taxa such as birds which have lower extrinsic mortality risk in early life, it may pay females to invest highly in the production of relatively few, high quality offspring. Indeed, female birds typically provision their eggs with far higher concentrations of antioxidants than sea turtles and other reptiles studied to date (**Chapter 2**); although whether this increased investment is associated with improved oxidative stress resistance in offspring requires further investigation.

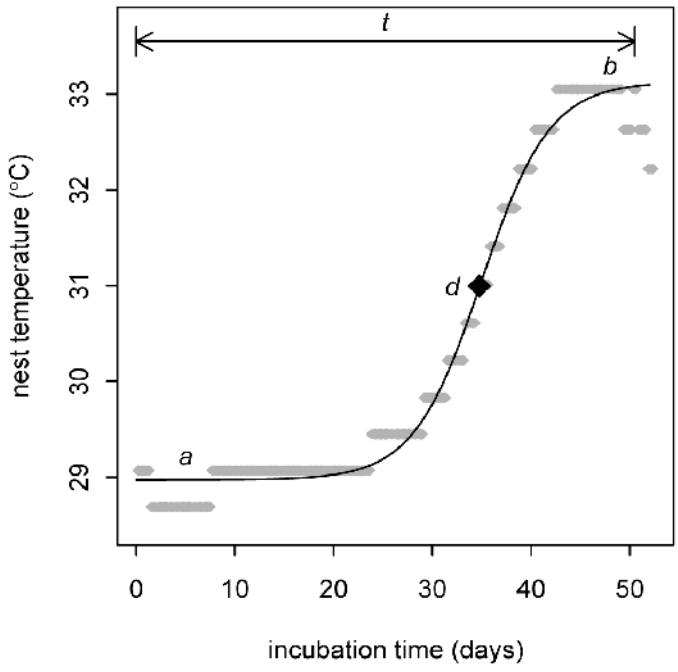
In conclusion, this study has shown that the nest environment provided by a female can have important consequences for oxidative stress in her offspring, however investments of fat-soluble antioxidants in eggs did not compensate for this risk. Studies of maternal carotenoid provisioning to eggs in birds and fish have similarly found weak or equivocal benefits for offspring health, vigor and survival (**Table 1.1**, pg. 4). Less is known about the ecological significance of vitamin E deposition in eggs, however the results of the present study indicate that variation in maternally-derived vitamin E has little effect on hatching success, or hatchling phenotypes in this species. The lack of strong carotenoid- and vitamin E-mediated maternal effects is perhaps surprising given the magnitude of variation among clutches often found in wild populations (3 – 30 fold; this study; Biard et al. 2005; Svensson et al 2006; Remes et al 2007; Saino et al. 2008; Isaakson et al 2008). Such variation may still have fitness consequences, for example by influencing immune function (Saino et al. 2003; Biard et al. 2005; Berthouly et al. 2008) and parasite resistance in offspring (Ewen et al. 2009), in addition to downstream effects on nutrient assimilation in later life which remain little studied (Koutsos et al 2003; Blount et al. 2003). However, it also seems possible that maternal transfer of variable levels of antioxidants into eggs may sometimes be functionally neutral, with no adaptive value for mother or offspring. The proximate mechanisms which regulate antioxidant deposition into eggs are poorly understood (see **Chapters 3 & 4**), although the non-specific transfer of inert and even toxic xenobiotics into egg yolk (Astheimer et al. 1989, de Solla et al. 2001) suggests that deposition of lipophilic compounds into eggs may often be a physiological inevitability.

## 5.6 Appendices: Supplementary methods and results

### 5.6.1. Nest temperature profiles during incubation of green turtle eggs

Incubation temperature during development in sea turtles is a function of sand temperature but also the metabolic heat produced by embryos in the latter half of development, which is proportional to clutch size (Broderick et al. 2001). In the present study, the change in nest temperature over the incubation period was well approximated by a four parameter logistic model with lower and upper asymptotes (non-linear least squares regression, all $p < 0.001$, > 96% of variation explained for all clutches; **Figure A5.5**). Post-hatching, there is a sharp decline in nest temperature caused by hatchlings leaving the nest chamber containing the temperature logger (**Figure A5.5**). This decline can therefore be used to estimate true incubation times (*t*) for eggs developing *in situ* (see § 5.6.2).

**Figure A5.5**. Representative profile showing the change in nest temperature across the incubation period in green turtle nests. The change is well approximated by a four parameter logistic function of the form $y = a + (b\text{-}a) / (1+e^{(c-x)/d})$, where *a* is the lower asymptote at the start of incubation before metabolic heating, *b* is the upper asymptote at the end of development, *c* is a scaling parameter and *d* is the inflection point (♦). The gradient at *d* reflects the fastest rate of increase in nest temperature due to metabolic heating. The decline in temperature after the upper asymptote reflects readings made post-hatching and can be used to estimate development times (*t*).



Lower asymptotic temperature early in development and mean temperature over the entire incubation period were not significantly related to clutch size or the number of surviving embryos (all $p > 0.2$), but were strongly predicted by laying date ($F_{1,29} = 30.4$, $p < 0.001$, $r^2 = 0.52$), due to the seasonal temperature gradient at Ascension Island (Godley et al. 2001). However, metabolic heating did influence nest temperatures later in development, as we found a significant relationship between clutch size and the rate of increase in nest temperature during incubation (gradient at the inflection point; multiple regression with laying date as covariate, $F_{1,29} = 21.0$, $p < 0.001$) and the asymptotic maximum temperature reached ($F_{1,28} = 8.0$, $p = 0.009$).

### 5.6.2. Estimating development times from nest temperature profiles

Sea turtles bury their eggs in deep nest chambers excavated in sandy beaches, which leads to an interval separating hatching and emergence of hatchlings from the nest ('emergence lag'; reviewed in Godfrey & Mrosovsky 1997). However, it is possible to reliably estimate true development times *in situ* from the rapid decline in nest temperature profiles post-hatching (**Figure A5.5**).

According to Gillooly et al. (2002), temperature is the principle environmental determinant of development times in ectotherms due to its effect on the rate metabolic reactions. Using a theoretical model based on first principles of kinetics they predicted that log-transformed incubation times (when corrected for body mass among species) should vary as a universal linear function of $T_c/(1 + (T_c/273)$, where $T_c$ is temperature (ºC), with a slope of $\alpha = -\overline{E}/kT_0^2$ where $\overline{E}$ is the activation energy, $k$ is the Boltzmann constant and $T_0 = 273$ ºC. Moreover, given that the rate of ontogenetic development is fundamentally dependent on metabolism, using the average activation energy for metabolic reactions ($\overline{E} = 0.6$ eV) they predicted that the slope of the line should have a universal value of $\alpha = -0.093$ per ºC. Substituting estimated incubation times (as described above) and mean nest temperatures over this period for green turtle clutches into the model of Gillooly et al. (2002) produced a strong linear relationship with a slope of $\alpha = -0.10$ per ºC (**Figure A5.6**). The closeness of this value to the theoretical slope suggests that indirect estimates of incubation times from nest temperature profiles in green turtles are reliable. Furthermore, it confirms that developmental rates in green turtles are strongly determined by the effect of nest temperature on metabolic reactions.

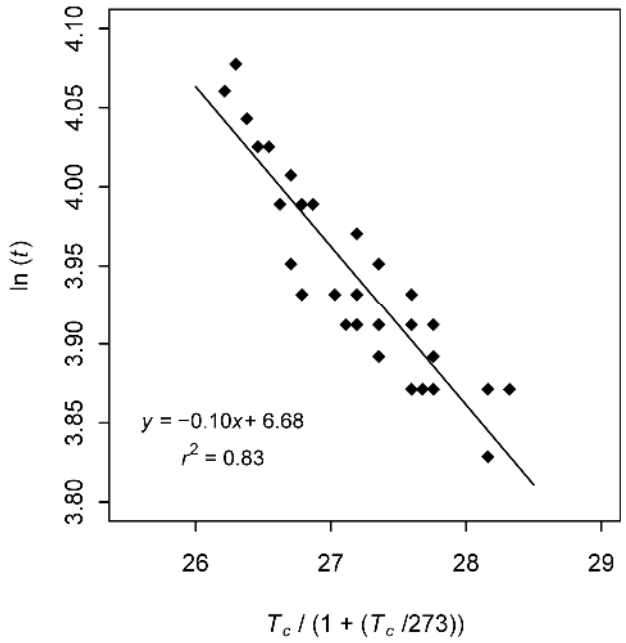



**Figure A5.6.** Relationship between mean nest temperature ($T_c$) and incubation time ($t$) of green turtle clutches ($N$ = 29). Incubation time was estimated from nest temperature profiles (see Figure A5.5) and nest temperature is given as $T_c/(1 + (T_c/273)$, following Gillooly et al. (2002) (see text). The line was fitted by least-squares linear regression.

Estimated incubation times were highly correlated with total times from oviposition until first hatchling emergence from the nest (Pearson's product-moment correlation, $r$ = 0.81, d.f. = 28, $p < 0.001$). The mean emergence lag separating estimated hatching time and first emergence from the nest (mean ± SE: 5.3 ± 0.4 days, range: 2 – 7 days) was within the range reported previously in sea turtles (4-7 days; summarised in Godfrey & Mrosovsky 1997), and was influenced by both incubation time and by the nest environment. Hatchlings with shorter incubation times took longer to emerge from the nest post-hatching (multiple regression, $F_{1,26}$ = 25.0, $p < 0.001$), and emergence lag was longer for hatchlings in deep nests ($F_{1,26}$ = 21.7, $p < 0.001$) and where the volumetric water content of the sand was higher ($F_{1,26}$ = 10.9, $p$ = 0.003), presumably due to the longer and more compacted sand column hatchlings were required to dig through (see also Van de Merwe et al. 2005). Mean incubation temperature did not predict emergence lag from nests in a model that also included incubation time ($F_{1,25}$ = 0.09, $p$ = 0.77).

# **Chapter 6** Home is where the heat is: natal homing and local adaptation to thermal regimes in the green turtle.

## 6.1. Abstract

Marine turtles are renowned for their ability to return to their birthplace in order to breed ('natal homing'), yet the adaptive significance of this behaviour remains enigmatic. One theory suggests that natal homing may increase reproductive success through the accumulation of habitat-specific adaptations, although empirical support for this hypothesis is currently limited to anadromous fishes. Using a combination of observational and common-garden approaches, I investigated whether embryonic thermal tolerances are locally adapted to the temperature of specific nesting beaches in a population of green turtles (*Chelonia mydas*) where females display strong fidelity to their natal sites. As predicted, the offspring of females nesting on a naturally hot beach had markedly improved viability and grew larger at high developmental temperatures compared to the offspring of females nesting on a cooler, neighbouring beach. This disparity was not related to maternal resource provisioning in eggs (egg size, lipids, water, antioxidants, and fatty acids). Combined with previous genetic studies in the same population, these results suggest that natal homing can lead to adaptive divergence in fitness enhancing traits in sea turtles and thus highlight a potentially unifying mechanism underpinning the evolution of homing behaviour. From a conservation perspective, where breeding populations are locally adapted to specific thermal regimes there are likely to be significant implications for their viability in the face of climatic change.

## 6.2. Introduction

The tendency for animals to return to their natal sites in order to breed ('natal homing') is phylogenetically widespread and is responsible for some of the most iconic migrations in the natural world (Meylan et al. 1990; Quinn & Dittman 1990; Dingle 1996; Brown & Shine 2007; Rooker et al. 2008; **Chapter 1**). The best known of these species, the sea turtles and the anadromous salmon, frequently migrate thousands of kilometres to reach their natal breeding grounds, guided by a combination of geomagnetic and olfactory cues imprinted during early life (Meylan et al. 1990; Quinn & Dittman 1990; Lohmann et al. 2008). However, why animals undertake such epic journeys to reproduce where they themselves were born is enigmatic. Homing migrations are energetically costly and potentially hazardous, yet migrants often bypass apparently suitable breeding habitat used by conspecifics (Lohmann et al. 2008). Such extraordinary behaviour could be explained if individuals are locally adapted to their natal sites (Quinn & Dittman 1990). Natal homing restricts gene flow between breeding populations and may therefore promote rapid divergent selection for traits which increase reproductive success in specific environments (Hendry et al. 2000). Indeed, breeding aggregations of salmon are often genetically distinct and display a multitude of behavioural and morphological adaptations to their natal spawning grounds, with resident fish generally outperforming immigrants or transplants from other sites (Quinn & Dittman 1990; Taylor 1991; Hendry et al. 2000). However, whether natal homing is necessarily associated with local adaptation is unclear.

In sea turtles, while it is generally accepted that homing allows females to locate a nest site which successfully produced offspring in the previous generation (Lohmann et al. 2008), the hypothesis that individuals are locally adapted to their specific natal habitats has never been tested. Unlike salmon, which may reside at their natal sites for several years (Dittman & Quinn 1996), sea turtles interact with their natal habitat for only a tiny fraction of their life cycle, with newborn hatchlings dispersing rapidly and not returning for decades (Carr 1967). Moreover, whilst homing has led to genetic divergence among breeding aggregations along maternal lineages, population genetics has revealed considerable male-mediated gene flow at local and even regional scales (Lee et al. 2007; Bowen & Karl 2007) which should tend to disrupt local adaptation (Kawecki & Ebert 2004). Nonetheless, nesting sites also vary in characteristics which might be expected to

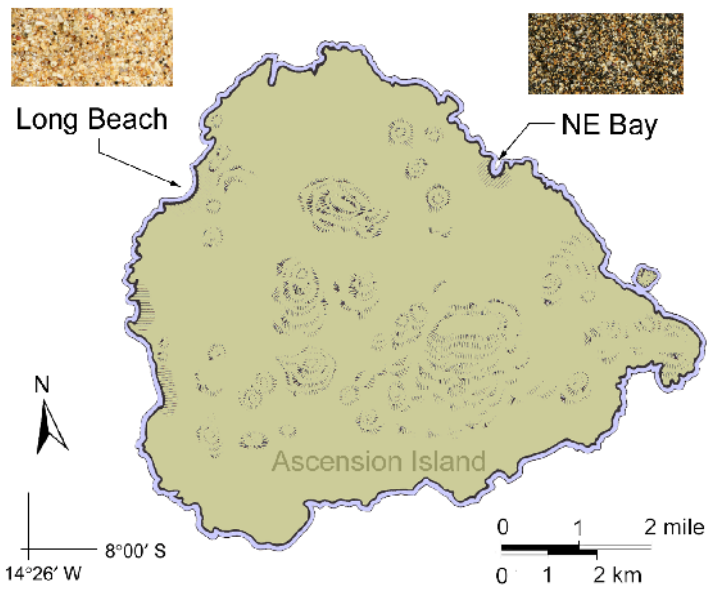



**Figure 6.1.** Map of Ascension Island showing nesting beach locations and sand color of Long Beach (LB) and North East Bay (NEB) photographed under standardized conditions of lighting and exposure *(map modified from Carr 1967)*.

produce divergent selection. For example, sea turtles bury their eggs on sandy beaches where offspring phenotypes and survival are profoundly affected by the local microclimate and physical attributes of the beach, most notably incubation temperatures (Ackerman 1997). Provided these local characteristics are stable over generational timescales, natal homing may ensure a consistent developmental environment for natural selection to drive adaptive adjustments in embryonic tolerances.

We tested whether green turtles are locally adapted to their natal sites at Ascension Island in the South Atlantic Ocean; the rookery which first prompted Carr's theory of natal homing in marine turtles (Carr 1967). Nesting at Ascension Is. is distributed over a mixture of biogenic and volcanic sand beaches which vary dramatically in sand color and albedo (reflectance of solar radiation), resulting in significant heterogeneity in incubation temperatures among nesting sites, despite the island's small size (**Figure 6.1**) (Hays et al. 2001, 2003). Females display strong site fidelity to specific beaches within and among years (Mortimer & Portier 1989) and genetic profiling has revealed matrilineal divergence between Ascension's primary nesting sites (Long Beach and North East Bay), consistent with precise natal homing (Formia et al. 2007; Lee et al. 2007). Long Beach (LB) and North East Bay (NEB) differ markedly in sand color and incubation temperatures (**Figure 6.1;** Hays et al. 2003), thus we predicted that natal

homing would lead to local adaptation of embryonic tolerances to these contrasting thermal regimes.

## 6.3 Results

In 2004 we conducted an observational study using *in situ* nests to define the natural range of incubation temperatures for LB and NEB and to determine how temperature influences clutch survival at each site (data collected by A.C. Broderick & B.J. Godley). Nests on NEB were on average 2.2 ºC hotter and there was very little overlap in temperatures experienced between sites (NEB: mean = 32.4 ± 0.1 ºC, range = 31.5 – 33.6; LB: mean = 30.2 ± 0.1 ºC, range = 29.2 – 31.6). The proportion of eggs hatching decreased at hotter incubation temperatures at both beaches (GLM, $F_{1,58} = 56.0$, $p < 0.001$), however clutches laid at NEB had higher hatching success at a given temperature compared to LB clutches, suggesting an increased upper thermal tolerance limit for NEB eggs ($F_{1,58} = 9.3$, $p = 0.004$; beach × temperature interaction NS, $F_{1,57} = 1.2$, $p = 0.27$; **Figure 6.2A**).

To determine whether offspring are thermally adapted to their natal beaches, in 2008 we performed a common-garden experiment using artificial incubators to replicate incubation temperatures from *in situ* nests at LB (29 ºC) and NEB (32.5 ºC) whilst holding other environmental variables constant (incubation substrate, water potential, aeration): hereafter 'cool' or 'hot' treatments respectively (see Materials and Methods § 6.5). We collected eggs from nesting females at LB and NEB and split them between incubation treatments ($N = 3$ eggs per female per treatment) to assess the relative performance of developing embryos from each site in both cool and hot environments (this effectively simulates the phenomenon of females straying between beaches). Consistent with the results of the *in situ* observational study, eggs laid on NEB had significantly improved hatching success in the hot treatment compared to those from LB (proportion eggs hatched, NEB: 53%, LB: 17%; likelihood-ratio test, $\chi_1^2 = 6.9$, $p = 0.008$; **Figure 6.2A**); although in the cool treatment hatching success was universally high for eggs from both beaches (97 % in each case; $\chi_1^2 = 0.15$, $p = 0.70$) (see Materials and Methods § 6.5). Examination of unhatched eggs from the hot treatment revealed a bimodal distribution in the stages of embryonic mortality, with either no visible embryos ('early-stage' mortality; all eggs were fertile), or large, fully formed embryos present ('late-stage' mortality) (see Materials and Methods § 6.5). The proportion of

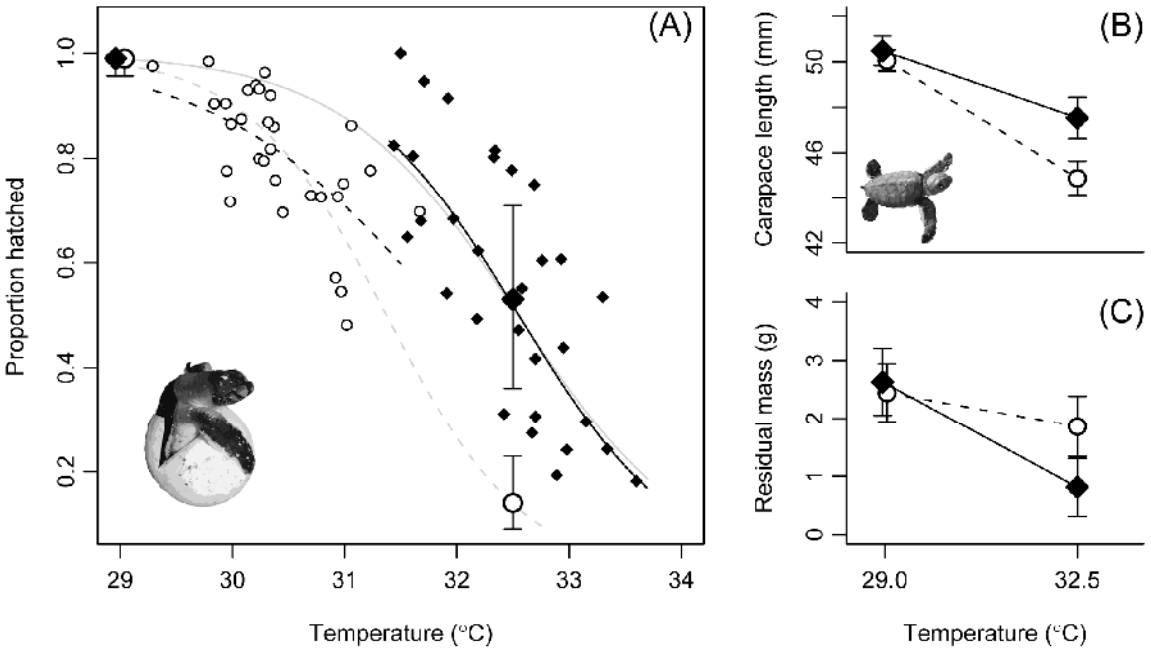



**Figure 6.2.** Effects of incubation temperature on hatching success and hatchling morphology for clutches laid at Long Beach (LB: ○, dashed lines) and NE Bay (NEB: ♦, solid lines). (A) Small symbols show hatching success of *in situ* nests across a natural range of incubation temperatures with lines in bold fitted using logistic regression. Large symbols show mean hatching success (estimate ± 1SE from binomial GLMM) from a common-garden experiment where eggs were incubated at either 29 ºC ('cool') or 32.5 ºC ('hot'). Faded lines were fitted using logistic regression with data from *in situ* nests and constrained to pass though estimates from the common-garden experiment. (B) and (C) show effects of origin and incubation temperature on hatchling straight carapace length (SCL) and residual body mass (controlling for SCL) respectively in the common-garden experiment (estimates ± 1 SE from GLMM).

eggs containing early-stage embryos was identical for both beaches (20 % in each case; binomial GLMM, effect of origin: $\chi_1^2 = 0.001$, $p = 0.98$), so the disparity in hatching success we observed in the hot treatment was due to a significant increase in late-stage embryonic mortality for eggs laid at LB compared to NEB (proportion of eggs, NEB: 27 %, LB: 63 %; $\chi_1^2 = 8.2$, $p = 0.004$; **Figure 6.3**).

In addition to effects on embryo survival, we also examined how different thermal regimes influenced development times and hatchling morphology. Eggs developed faster in the hot treatment (days to hatching, hot: mean = 43.9 ± 0.2 d, cool: mean = 53.8 ± 0.2 d), as is typical in reptiles (Birchard 2004), but development times were comparable for eggs laid at LB and NEB (likelihood ratio test, effect of treatment: $\chi_1^2 =$

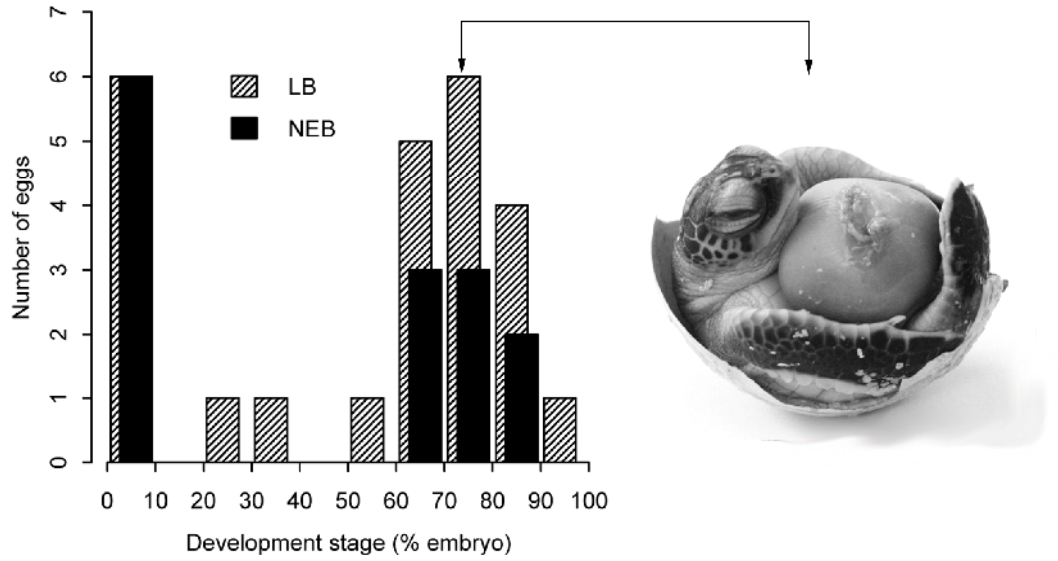



**Figure 6.3.** Stages of embryonic mortality for unhatched eggs from Long Beach (LB) and North East Bay (NEB) in the hot incubation treatment (32.5 ºC). Developmental stage is expressed as the mass of the yolk free embryo as a proportion of total egg contents (i.e. embryo + yolk sac). The photograph shows a representative embryo at 70-80% development ('late-stage' mortality) with yolk sac attached.

257, $p < 0.001$; effect of beach: $\chi_1^2 = 0.71$, $p = 0.40$; interaction: $\chi_1^2 = 0.04$, $p = 0.84$). However, despite similar hatchling morphologies for both beaches in the cool treatment, NEB hatchlings from the hot treatment were larger than LB hatchlings (straight carapace length; SCL), yet were lighter for their size (residual mass controlling for SCL), indicating that growth trajectories were differentially affected by high incubation temperatures (origin × treatment interaction: SCL, $\chi_1^2 = 5.9$, $p = 0.015$; residual mass, $\chi_1^2 = 4.4$, $p = 0.035$; **Figures 6.2B** and **6.2C**). Sex is also temperature-dependent in marine turtles; however hatchlings from both NEB and LB are exclusively female at an incubation temperature of 32.5 ºC (Godley et al. 2002). Thus, the differences in performance we observed in the hot treatment cannot be explained by sex-biased viability and growth.

While our results are consistent with an adaptive increase in the upper thermal tolerance limits of NEB embryos, we also considered the possibility that embryonic thermal tolerances may be shaped by non-genetic maternal effects mediated through resource provisioning in eggs. However, we found no support for this explanation. A sample of eggs ($N = 3$) from each clutch used in the common-garden experiment was analyzed for

constituents which might be expected to modify embryonic thermal tolerances, i.e. saturated/unsaturated fatty acids, antioxidant compounds and energetic reserves/egg size (Hazel & Williams 1990; Pörtner 2002; Martin 2008). There were no significant differences in masses of eggs, yolk, albumen and lipid, concentrations of vitamin E and carotenoids (maternally-derived antioxidants), or arcsine-transformed proportions of saturated, polyunsaturated or monounsaturated fatty acids in yolk lipids for clutches laid at LB and NEB (unpaired *t*-tests, all $t < 1.4$, all $p > 0.19$; Appendix Table A6.1). Yolk fatty acid profiles were also reduced to a smaller number of uncorrelated variables using principle components analysis (PCA) following a centred log-ratio transformation to account for the compositional nature of the data (van den Boogaart & Tolosana-Delgado 2008). PC scores on the major axes (eigenvalues > 1; PC1: 32%, PC2: 24%, PC3: 13%) did not differ significantly for clutches laid at NEB and LB (MANOVA, $F_{3,16} = 0.99$, $p = 0.42$; Appendix Table A6.1) indicating that yolk fatty acid compositions were comparable for both beaches.

## 6.4. Discussion

The results of this study indicate that, as in anadromous fish, natal homing in marine turtles can lead to adaptive divergence in fitness enhancing traits. Using a combination of *in situ* and experimental approaches, we found that the offspring of females nesting on a naturally hot beach (NEB) had significantly improved viability and grew larger (a potential determinant of early survival in sea turtles; Gyuris 2000) at high incubation temperatures compared to the offspring of females nesting on a neighbouring cooler beach (LB; **Figures 6.2A** and **6.2B**). This disparity is unlikely to be the result of egg-mediated maternal effects because in a detailed analysis of egg composition we found no significant differences between nesting sites. Indeed, to our knowledge there is little empirical evidence of egg composition modifying offspring thermal performance in any species (with the possible exception of egg size; Martin 2008). Given that females nesting at LB and NEB show strong beach fidelity and genetic differentiation between sites (Mortimer & Portier 1989; Lee et al. 2007; Formia et al. 2007), our results suggest that there has been an adaptive increase in the upper thermal tolerance limits of NEB embryos in response to the hotter developmental environment of this beach.

Various mechanisms of thermal adaptation have been proposed in ectotherms, including lipid membrane restructuring (Hazel & Williams 1990), shifts in aerobic scope (i.e. the

capacity to maintain aerobic respiration at different temperatures and rates of metabolism; Pörtner 2002) and modification to enzyme thermal stability (Angilletta 2009). Although we did not explicitly test such mechanisms, an increase in embryonic aerobic scope at high temperatures is a likely candidate to explain the superior performance of NEB eggs in hotter environments. Significant differences in viability between NEB and LB embryos in the hot treatment were only apparent late in development (**Figure 6.3**) when rates of growth and oxygen consumption are at a maximum (Booth 1998), as would be expected if aerobic capacity determined survival. Furthermore, oxygen-deprived reptilian embryos typically hatch smaller with larger residual yolk sacs due to metabolic depression and impaired yolk utilization (Owerkowicz et al. 2009), so the reduced size and increased residual body mass of LB hatchlings at high temperatures is consistent with oxygen-limited growth compared to locally adapted NEB hatchlings (**Figure 6.2C**).

Our findings are particularly remarkable given the close proximity of NEB and LB (6 km apart, relative to a 2000 km migration; **Figure 6.1**; Meylan et al. 1990) and significant male-mediated gene flow within the Ascension population (Lee et al. 2007) which should tend to oppose local adaptation (Kawecki & Ebert 2004). In fact, given that sex is temperature dependent in sea turtles and incubation times at NEB are predominantly feminizing (Godley et al. 2002), almost all NEB females presumably mate with males hatched from an adjacent cooler beach. This raises the intriguing possibility that embryonic thermal tolerances have diverged along maternal lineages through maternally-inherited mitochondrial genes (sea turtles lack sex chromosomes). Indeed, the mitochondria play a pivotal role in defining oxygen-limited thermal tolerances in ectotherms (Pörtner 2002) and contain several protein-encoding genes involved in aerobic respiration (Boore 1999). Moreover, the mitochondrial genome has been identified as a target of selection in thermally heterogeneous environments and may be locally adapted to specific thermal regimes (Cheviron & Brumfield 2009). Thus, our results suggest that local adaptations in offspring thermal tolerances can arise through maternal natal homing in the face of male-mediated gene flow.

The evolutionary basis of natal homing must ultimately lie in the increased reproductive success of individuals which return to breed at their natal sites relative to those that attempt to breed elsewhere (Quinn & Dittman 1990; Brown & Shine 2007; Lohmann et al. 2008). Local adaptation could therefore underpin the evolution of homing behavior

by widening the disparity in reproductive success between the natal site and possible alternatives (see **Chapter 8.2**). In this respect, green turtles nesting at NEB present an apparent paradox, in that the majority of NEB females remain faithful to this site (Mortimer & Portier 1989) despite their offspring performing better in the cool (LB) treatment than the hot (NEB) treatment (**Figure 6.2**). However, the selective advantages of homing must be viewed within the overall context of the breeding habitat. Nesting at Ascension Island is not limited to LB and NEB (the primary nesting sites in terms of the numbers and density of nests; Godley et al. 2001); there are some 30 additional sandy beaches and coves, many of which are rocky, desiccating or prone to inundation by the sea (Mortimer 1990), which is likely to result in high overall failure rates for exploratory nesting. Moreover, NEB may have historically been a highly successful nesting site for locally-adapted individuals. Climate reconstructions for Ascension Is. indicate a 0.5 ºC rise in average beach temperatures over the past 150 years (~ 4-6 turtle generations; Hays et al. 2003). Based on contemporary thermal performance curves, this suggests that mean hatching success at NEB may have historically stood at 72 %, close to present-day levels at LB (mean = 80 %; **Figure 6.2A**). The apparent inferiority of NEB as a nesting site compared to LB may therefore be a recent phenomenon, reflecting a failure of embryonic tolerances to evolve in step with recent climate warming.

The pervasive effects of temperature on marine turtle reproduction (e.g. sex determination, embryonic viability) coupled with the relative inflexibility of natal homing has raised serious concerns for the persistence of breeding populations under current climate warming projections (Hawkes et al. 2007; Fuentes et al. 2010). Whilst we have shown that embryonic tolerances can adapt to specific thermal regimes, such evolutionary adjustments are unlikely to keep pace with the decadal scale of anthropogenic climate warming. Indeed, the possibility of widespread thermal adaptation should be accounted for in models predicting sea turtle population responses to global climate change (Hawkes et al. 2007; Fuentes et al. 2010), since even modest warming is likely to cause declines in reproductive success at individual nesting sites as sand temperatures exceed locally-adapted developmental tolerances. However, at the population or species level it is possible that locally-adapted, thermotolerant lineages will confer some degree of resilience to the effects of climate change (as suggested for corals; Hughes et al 2003), insofar as breeding site fidelity is not absolute (Bowen & Karl 2007; Lohmann et al. 2008) facilitating the spread of favorable genes.

## 6.5. Materials and Methods

**6.5.1 Study site.** The study was carried out at Ascension Island, UK - an isolated volcanic peak in the South Atlantic Ocean which hosts one of the largest Atlantic breeding populations of green turtles (Broderick et al. 2006; **Figure 6.1**). Nesting occurs between January and June, with a majority of the activity focussed at a small number of primary nesting locations (Godley et al. 2001). Long Beach (LB) and North East Bay (NEB) support the highest numbers and density of nesting females on the island (36 % and 12 % respectively; Godley et al. 2001) but provide very different developmental environments for eggs: NEB is composed of black volcanic sand and is on average 2.8 ± 0.08 ºC hotter at nest depth (~ 70 cm) than LB which has paler sand with a high proportion of biogenic material (**Figures 6.1 and 6.2A**)

**6.5.2 Temperature and hatching success *in situ*.** In 2004 an observational study was undertaken to relate the hatching success of *in situ* clutches to nest temperatures at LB and NEB. Females nesting at LB and NEB were selected at random between 22$^{nd}$ February and 7$^{th}$ March 2004 ($n$ = 30 per beach). An archival temperature logger (Tinytalk, Gemini Data Loggers, Chichester, UK; precision of 0.3 ºC) was placed into the center of each clutch during oviposition and was programmed to record nest temperature at 4-hour intervals throughout the incubation period. Loggers were recovered during nest excavation following hatchling emergence and hatching success was estimated from the number of hatched and unhatched eggs.

**6.5.3. Common-garden rearing experiment.** In 2008 we conducted a common-garden experiment using artificial incubators to replicate the thermal regimes of *in situ* nests on LB and NEB. Artificial incubation was carried out in custom-built forced air incubators, constructed of expanded polystyrene and controlled by pulse-proportional thermostats. Temperatures were set at either a constant 32.5 ºC (mean incubation temperature of *in situ* nests on NEB) or a constant 29 ºC (lower extent of the range of incubation temperatures for *in situ* nests on LB; see also Godley et al 2002); hereafter, 'hot' and 'cool' treatments, respectively. The temperature of the cool treatment was chosen in order to maximise the variation in offspring development and survival; we did not set the temperature of hot incubators above the mean of NEB nests due to the risk of causing complete mortality of eggs laid at both sites (see **Figure 6.2A**). Internal incubator temperatures were monitored continuously using Tinytalk dataloggers (cross-

calibrated against a NIST certified mercury thermometer) and remained within ± 0.3 ºC of nominal values throughout incubation.

Eggs were collected during oviposition from females nesting at LB and NEB between 18th and 28th March 2008 ($N$ = 10 clutches per beach), corresponding with the period of peak nesting activity at Ascension Is. (Godley et al. 2001). Pairs of eggs were sampled from the approximate start, middle and end of the laying sequence of each clutch and one per pair allocated at random to either the hot or cool incubation treatment within one hour of oviposition (giving $N$ = 3 eggs per clutch per treatment). Eggs were incubated half buried in moist vermiculite (hydrated to constant water potential of ~ –50 kPa throughout incubation; as recommended by Booth (2004)) in individual plastic containers sealed with loosely fitting lids to maintain humidity, and perforated with a standard number of holes to permit gas exchange. Open trays of water were also placed inside incubators to create a humid atmosphere and incubators were aerated by opening for 5 × 1 minute daily. Incubators were closely monitored to determine hatching dates and eggs that failed to hatch were dissected to determine the stage of embryonic mortality (expressed as the mass of embryonic tissue as a proportion of total egg contents; see **Figure 6.3**). All eggs were fertile, as evidenced by 'chalking' on the upper shell surface at the site of vitelline membrane attachment (Booth 2004). Within 24 hours of emergence, surviving hatchlings were weighed (± 0.2 g) and straight carapace length (SCL) was measured using a digital calliper (± 0.1 mm) as an index of body size. By this time hatchling carapaces had fully straightened and residual yolks had been internalised into the plastron.

**6.5.4 Analyses of egg composition.** Three eggs were sampled for compositional analysis from each experimental clutch, one following collection of each pair of eggs for the incubation experiment (see § 6.5.3). Within one hour of collection eggs were carefully separated into their constituent parts (albumen, yolk) and weighed. The yolk portion was homogenised and stored at - 45 ºC awaiting biochemical analysis. Total lipids were extracted from an aliquot of yolk by homogenisation in chloroform/methanol (2:1, v/v) and lipid content determined gravimetrically after evaporation of the solvent (Folch et al. 1957). The fatty acid composition of a portion of the lipid extract was then analysed by gas chromatography/mass spectrometry (GC/MS) following derivitization to form fatty acid methyl esters (FAMEs), as described by

Craven et al. (2008). FAMEs were separated using a TraceMS instrument (ThermoQuest, Hemel Hempstead, UK) fitted with a Factor Four VF23-MS fused silica capillary column (high cyanopropyl modified methyl polysiloxane; Varian Chrompack, 60 m × 0.32 mm, 0.15 µm film thickness). A two-step temperature programme was used from 40 ºC (held for 2 min) to 100 ºC at 13 ºC/min, and then at 3 ºC/min to 260 ºC (held for 10 min) with helium as the carrier gas (flow rate = 2 ml/min). Peaks were identified by comparison with the retention times of standard FAME mixtures (Supelco, Bellefonte, PA) and peak areas integrated to express amounts of individual compounds as a proportion of total fatty acids. Double-bond positions of polyenoic fatty acids were assigned based on the elution order of positional isomers and by reference to their mass spectra (Fellenberg et al. 1987). GC/MS of fatty acids was performed by A. Kuhl at the Life Sciences Mass Spectrometry Facility, University of Bristol, UK.

Concentrations of vitamin E and carotenoids (fat-soluble antioxidants) in eggs were assayed as described in **Chapters 2 & 3**.

**6.5.5 Statistical analysis.** The hatching success of *in situ* clutches (binomial variable: number hatched / number unhatched) was modelled as a function of mean incubation temperature using generalized linear modelling (GLM) with a quasibinomial error structure (to account for binomial overdispersion) and with beach included as a fixed factor. Significance of the explanatory variables was evaluated using type III *F*-tests following deletion from the model, starting with the beach × temperature interaction.

The hatching success of eggs in the common-garden experiment was analyzed using two approaches. First, I analyzed hatchability as a binary response variable (hatched or not-hatched) using a generalized linear mixed model (GLMM), with treatment and beach of origin as fixed factors, clutch included as a random factor, and a binomial error structure with a logit-link function. Model simplification indicated a strong treatment effect (likelihood ratio test, $\chi_1^2 = 62.9$, $p < 0.001$) and higher hatching success for eggs laid on NEB ($\chi_1^2 = 6.2$, $p = 0.013$), although the origin × treatment interaction was non-significant ($\chi_1^2 = 1.4$, $p = 0.24$). This implies that NEB eggs had consistently higher hatchability across incubation treatments, despite identical numbers of hatched eggs from both origins in the cool treatment (29/30 eggs hatched in each case), indicating a problem with model fit. Indeed, an analysis subsetted by treatment confirmed a strong

effect of origin on hatching success in the hot treatment (likelihood ratio test, $\chi_1^2 = 6.9$, $p = 0.008$) while there was no difference between origins in the cool treatment ( $\chi_1^2 = 0.15$, $p = 0.70$). Secondly, therefore, I calculated the proportions of hatched eggs from each clutch within a treatment and used the arcsine square-root transformed values as the response variable in a GLMM with Gaussian errors. Model simplification indicated a significant origin × treatment interaction for hatching success (likelihood ratio test, $\chi_1^2 = 5.9$, $p = 0.015$).

Hatchling size (SCL) and residual body mass (controlling for SCL) in the common-garden experiment were analysed using GLMM with treatment and beach of origin as fixed factors, clutch included as a random factor, and a Gaussian error structure. Significance of the fixed effects was assessed using likelihood-ratio tests (compared against a $\chi^2$ distribution) following deletion from the model, starting with the origin × treatment interaction (alpha = 0.05). All analyses were carried out using R v. 2.9.2 (R Development Core Team 2009).

## 6.6. Appendix: Supplementary Results

**Table A6.1.** Composition of green turtle eggs from Long Beach (LB) and North East Bay (NEB) clutches used in the common-garden experiment. Values are expressed as mean ± 1 SE of 10 clutches per beach ($N$ = 3 eggs per clutch). Differences between beaches are assessed using Student's $t$ tests. Yolk fatty acid profiles were compared by MANOVA using major principle components (PCs) from a principle component analysis.

| Component | LB | NEB | $t$ | $p$ |
|---|---|---|---|---|
| *Egg components (g)* | | | | |
| Whole egg | 52.6 ± 1.3 | 52.6 ± 1.7 | 0.004 | 0.99 |
| Yolk | 19.9 ± 0.5 | 18.9 ± 0.5 | 1.35 | 0.19 |
| Albumen | 30.1 ± 1.5 | 31.2 ± 1.6 | 0.47 | 0.65 |
| Lipid | 3.65 ± 0.1 | 3.73 ± 0.1 | 0.48 | 0.50 |
| *Antioxidants (µg $g^{-1}$ yolk)* | | | | |
| Vitamin E | 51.1 ± 3.7 | 44.8 ± 6.3 | 0.86 | 0.40 |
| Carotenoids | 4.2 ± 0.3 | 4.2 ± 0.4 | 0.03 | 0.98 |
| *Fatty acids (% total fatty acid)* † | | | | |
| *Saturated fatty acids (SFA):* | | | | |
| 12:0 | 7.9 ± 0.5 | 7.3 ± 0.7 | | |
| 14:0 | 5.6 ± 0.2 | 5.2 ± 0.4 | | |
| 16:0 | 16.2 ± 0.4 | 16.1 ± 0.6 | | |
| 18:0 | 5.2 ± 0.3 | 4.7 ± 0.4 | | |
| *Monounsaturated fatty acids (MUFA):* | | | | |
| 14:1n-5 | 0.2 ± 0.02 | 0.2 ± 0.02 | | |
| 16:1n-7 | 5.9 ± 0.2 | 5.8 ± 0.4 | | |
| 18:1n-9 | 51.4 ± 1.1 | 53.5 ± 1.6 | | |
| 20:1n-9 | 0.3 ± 0.04 | 0.3 ± 0.03 | MANOVA on PCs 1-3; $F_{3,16}$ = 0.99, $p$ = 0.42 | |
| *Polyunsaturated fatty acids (PUFA):* | | | | |
| 18:2n-6 | 0.6 ± 0.08 | 0.6 ± 0.09 | | |
| 20:2n-9 | 0.3 ± 0.06 | 0.3 ± 0.03 | | |
| 20:3n-6 | 0.2 ± 0.03 | 0.1 ± 0.01 | | |
| 20:3n-9 | 0.2 ± 0.05 | 0.4 ± 0.06 | | |
| 20:4n-6 | 4.0 ± 0.2 | 3.2 ± 0.2 | | |
| 20:5n-3 | 0.4 ± 0.03 | 0.6 ± 0.07 | | |
| 22:4n-6 | 0.4 ± 0.05 | 0.4 ± 0.05 | | |
| 22:5n-3 | 0.9 ± 0.06 | 1.0 ± 0.09 | | |
| 22:6n-3 | 0.2 ± 0.03 | 0.2 ± 0.04 | | |
| Σ SFA | 35.0 ± 1.2 | 33.3 ± 1.8 | 0.78 | 0.44 |
| Σ MUFA | 57.8 ± 1.5 | 60.0 ± 2.1 | 0.90 | 0.38 |
| Σ PUFA | 7.1 ± 0.4 | 6.7 ± 0.4 | 0.85 | 0.41 |

† Fatty acids comprising < 0.1 % of total are not shown. Shorthand notations used for fatty acids specify the number of carbons and the number of double bonds, followed by the position of the terminal double bond relative to the hydrocarbon end of the molecule.

# **Chapter 7** Metabolic effects on the rate of egg production in marine turtles: implications for maternal behaviour

## 7.1. Abstract

In many ectotherms, the rate at which females can produce eggs is profoundly affected by ambient temperature and may be an important factor determining the costs of reproduction. According to the Metabolic Theory of Ecology (MTE), the rates of many biological processes should vary as a universal function of temperature, governed by the thermodynamics of enzyme catalysed reactions. However, despite broad empirical support, this prediction has not yet been tested with regards to egg production. In this chapter I show that the rate of egg production in marine turtles is indeed determined largely by the effects of water temperature on metabolic processes, whilst being unrelated to maternal phenotype and reproductive investment. In a longitudinal study of nesting green turtles (*Chelonia mydas*), the time taken to produce successive clutches of eggs declined significantly with seasonally increasing water temperatures, but was not repeatable for individual females and did not vary according to maternal body size, clutch size, egg size or total clutch mass. Using a collated data set of water temperatures and inter-clutch intervals from several different populations and species of marine turtle, I further show that a single thermodynamic function can explain variation in the rate of egg production in marine turtles, with an activation energy (or temperature dependence) that is remarkably consistent with the universal value predicted by MTE. Since water temperature is the principle determinant of egg production rate in marine turtles, gravid females might be expected to adaptively increase their operating temperatures through behavioural thermoregulation in order to reduce the time invested in reproduction. Indeed, an examination of inter-nesting behaviour reported for globally distributed breeding populations suggests that 'maternal thermophily' may be widespread among marine turtles.

## 7.2 Introduction

In recent years, a growing awareness of global climate change has focussed interest on the pervasive effects of temperature on animal life histories. Ectothermic organisms are particularly sensitive to ambient temperature, which influences a diversity of traits including incubation times, growth rates, fecundity and longevity (reviewed in Gillooly et al. 2002; Birchard 2004; Angilletta 2009; Munch & Salinas 2009; **Chapters 5 & 6**). Many of the physiological effects of temperature can be attributed to metabolism, which sets the rate at which all organisms allocate resources into fundamental life history processes. According to the metabolic theory of ecology (MTE), variation in metabolic rate among organisms is explained largely by operating temperature, through its effects on biochemical kinetics, and body mass (Gillooly et al. 2001; Brown et al. 2004). Thus, for a given body mass, metabolic rate in all aerobic organisms is predicted to increase as a single, universal function of temperature (universal temperature dependence; UTD), with a slope defined by the activation energy of enzyme-catalysed reactions (Gillooly et al. 2001). Moreover, because metabolism underpins higher-order ecological processes such as growth and reproduction, many biological processes ranging from life history traits to ecosystem dynamics are expected to exhibit a similar UTD (Brown et al. 2004). While the MTE is controversial, these predictions have received considerable empirical support in both marine and terrestrial ecosystems (Duarte 2007; Lopez-Urrutia 2008; Munch & Salinas 2009).

In ectothermic organisms, the time required for females to produce eggs is highly dependent on body temperature (Carroll & Quirting 1993; Hirche 1997; Calbet & Augusti 1999; Hays et al. 2002b; Berger et al. 2008; Lourdais et al. 2008). Given the physiologically integrated nature of egg production, such effects are probably largely driven by changes in metabolic rate; however whether the rate of egg production conforms to the UTD predicted by metabolic theory has not yet been established for any species. This rate may determine important fitness outcomes, such as fecundity (e.g. Carroll & Quirting 1993; Berger et al 2008) or the time invested in reproduction and its associated costs to females e.g. increased predation risks, reduced foraging opportunities and altered hormonal states (Shine 1980; Williams 2005). If rapid egg production confers a selective advantage, reproductive females may be expected to adaptively increase their operating temperatures through behavioural modifications to

optimise their laying rate. Such 'maternal thermophily' is known to occur in lizards and snakes (Shine 2006; Lourdais et al. 2008); however, the squamata are unusual amongst reptiles in having significant embryonic development prior to oviposition (Andrews 2004), meaning thermophillic behaviour in these taxa may serve to optimise offspring developmental conditions *in utero* (Wapstra 2000; Shine 2006), rather than to promote egg production. However, a recent study has suggested that gravid marine turtles may also actively seek out warm micro-habitats (Schofield et al. 2009). Unlike squamate reptiles, chelonids lay their eggs at a very early stage in development (gastrulation; Miller 1985) suggesting that maintenance of optimal conditions for embryogenesis is unlikely to drive such behaviour and accelerating egg production may be a proximate explanation.

All species of marine turtle lay multiple clutches within a single nesting season (Miller 1997), and it is known that the interval separating successive clutches declines as a function of increasing ambient water temperature (Sato et al. 1998; Hays et al. 2002b). Since ovulation of the follicles for each clutch immediately follows the previous nesting attempt in sea turtles (Owens 1980; Licht et al. 1982), and eggs are laid at a fixed developmental stage (Miller 1985), such 'nesting interval lengths' can be used as a robust measure of the time required to produce mature eggs. Comparative analyses have suggested that the relationship between nesting interval length and ambient temperature may be similar among species and populations of marine turtle (Hays et al. 2002b), as would be predicted if there is a common metabolic mechanism. However there is considerable residual, unexplained variation, which might suggest a phenotypic component to the rate of egg production.

In this chapter I first aim to determine the contributions of ambient temperature and maternal phenotypes to the rate of egg production by using repeated measures of nesting interval length taken for individual green turtles across a water temperature gradient. I then use a collated dataset of water temperatures and nesting interval lengths from different species and populations of marine turtle to test whether the temperature dependence of egg production is consistent with the predictions of MTE. Finally, since rapid egg production may help to reduce the costs of reproduction, I review evidence that female marine turtles actively adjust their operating temperatures to maximise their laying rate. I suggest that maternal thermophily may be a widespread phenomenon in marine turtles and that the relationship between temperature, metabolism and egg

production has therefore been important in shaping aspects of female reproductive behaviour.

## 7.3. Materials & Methods

### 7.3.1 Study site and field procedures

Nesting interval length data were collected for green turtles (*Chelonia mydas*) nesting at Ascension Island, South Atlantic Ocean (14°20′W, 7°55′S) during the 2007 breeding season. The study was conducted on Long Beach, which supports the highest density and numbers of nesting turtles on the island (Godley *et al.* 2001). To assess the contribution of maternal phenotype to the rate of egg production, we used radio-telemetry to make repeated measures of nesting interval length from individual females as they returned to lay successive clutches. VHF radio transmitters were affixed to the carapaces of a randomly selected sample of females ($N$ = 20) nesting between the 2nd and 12th of January, using a two-part epoxy resin. Females were also fitted with a PIT tag (Passive Integrated Transponder; Identichip, Animalcare Ltd., UK) implanted into the triceps muscle of the right fore-flipper to assist identification. Over 95% of nesting activity at Ascension Island occurs between January and May (Godley *et al.* 2001), so there is a high probability that tagged females were encountered whilst depositing their first clutch.

Nesting females were subsequently re-located using a scanning AR-8200 VHF receiver (AOR, Derbyshire, UK) and YAGI antenna (Biotrack, Dorset, UK.) during nightly patrols of the nesting beach (20:00 – 05:00) conducted at 1 h intervals. Females were observed from a distance to allow nest excavation and then approached to confirm the presence of eggs. Nesting interval length was then calculated as the number of days elapsed between nesting attempts. In some instances ($N$ = 5) emergent females returned to the sea without laying and subsequently nested on the following night. Since emergence implies that females are carrying mature eggs but that nesting is somehow interrupted, in these cases we measured nesting interval length as the time up until emergence (following Sato et al. 1998; Alvarado & Murphy 1999; Hays et al. 2002b). However, subsequent nesting intervals were measured relative to actual laying dates, as ovulation of the follicles for the next clutch immediately follows successful nesting in sea turtles (Licht et al. 1982).

Body mass has important effects on metabolic rate and thus on the rate of biological processes (Gillooly et al. 2001), but is logistically difficult to measure in adult marine turtles (e.g. Hays et al. 2002b). Thus, curved carapace length (CCL; ± 1 cm) was used as an estimator of body size in study females, as it is highly correlated with body mass in this population (Hays et al. 2002b). Since the size or number of eggs produced may also contribute to the time necessary to produce a clutch, mean egg mass (± 0.2 g) for each clutch laid by study females was calculated for a sample of 3 eggs collected from the 10$^{th}$, 50$^{th}$ and 10$^{th}$ from last positions in the laying sequence (to account for within-clutch variation in egg size; **Chapter 3**). The locations of all clutches were then marked and clutch size estimated post-hatching from the number of hatched and unhatched eggs.

### 7.3.2 Measuring water temperature

Mean water temperature for the duration of each observed nesting interval was estimated from nocturnal MODIS-Aqua sea surface temperature (SST) images (4km resolution; Goddard Space Flight Centre). Daily mean SST for a box of 1º latitude x 1º longitude centred on Ascension Island (14$^{o}$20′W, 7$^{o}$55′S) was extracted using MATLAB v7, and these daily data were used to calculate mean SST between the start and end date of each nesting interval. Whilst remotely sensed SST is an indirect measure of the thermal environment experienced by females, the relationship between water temperature and nesting interval length estimated in this study was very close to that estimated previously using animal-borne temperature loggers, suggesting that SST reliably reflected actual temperature experiences (see Results § 7.4.1).

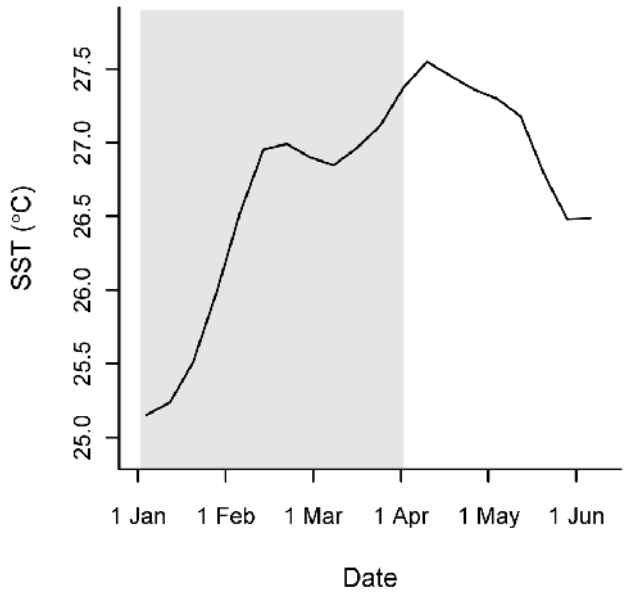



**Figure 7.1.** Sea surface temperature (SST) at Ascension Island during the 2007 green turtle nesting season (from nocturnal MODIS-Aqua satellite data). The shaded area shows the period during which the study was conducted.

### 7.3.3. Statistical analysis

A total of 46 inter-nesting intervals from 18 individual turtles were recorded over the course of the study (2 tagged females were never relocated). Generalised linear mixed models (GLMM) with female identity included as a random factor were used to test the effects of SST, clutch size, egg size and total clutch mass (clutch size × egg mass) on nesting interval length. Clutch characteristics could not be obtained for all nesting intervals as females were occasionally encountered during the post-laying, cover-up phase of nesting and a number of clutches were destroyed by tidal inundation or excavation by other turtles before clutch size could be determined. Analyses including measures of maternal reproductive investment were therefore performed using a subset of intervals for which clutch data was available ($N$ = 34 intervals from 15 females). The significance of fixed effects in GLMMs (SST and clutch characteristics) was assessed using likelihood ratio tests to compare the full model with a reduced model minus the effect of interest (Crawley 2007).

In order to estimate the overall proportion of variation in nesting interval length explained by phenotypic differences among females, I restricted the dataset to those individuals for which two or more measurements were made during the study and calculated the intra-class correlation coefficient, or repeatability score ($R$) using the equation $R = S^2_B / [S^2_B + S^2_E]$; where $S^2_B$ is the among-female variance component and $S^2_E$ is the within-female or residual variance component from a GLMM fit using restricted maximum likelihood (Nakagawa & Cuthill 2007; Zuur et al. 2009). SST was included in the model as a fixed effect. The significance of the among-female variance component was assessed by comparing a GLMM containing the random effect to a simple linear model fit by generalized least squares using a likelihood ratio test (as recommended by Zuur et al. 2009). Details of further statistical analyses are provided in the text of the results. All statistical analyses were performed using R v. 2.5.1 (R Development Core Team 2009).

## 7.4. Results

### 7.4.1 Determinants of nesting interval length.

Study females laid an average of 3.9 ± 0.3 clutches (mean ± SE) over the course of the nesting season separated by an average interval of 13.3 ± 0.2 days. Due to seasonal climatic fluctuations at Ascension Island, sea surface temperature (SST) increased

during the study period (**Figure 7.1**) and this had a profound effect on the length of the interval between successive clutches of individual turtles: nesting interval length declined significantly as water temperature increased (likelihood ratio test; $\chi_1^2 = 33.5$, $p < 0.001$, $N = 46$ intervals from 18 females; **Figure 7.2A**). In contrast, maternal phenotype and reproductive investment explained little of the observed variation in interval length. For turtles where two or more measurements were made ($N = 14$ females and 42 intervals), individual repeatability of nesting interval length was low and not statistically significant ($R = 0.03$, $\chi_1^2 = 0.013$, $p = 0.91$; **Figure 7.2B**). Consistent with the low repeatability of nesting interval length, there were no significant relationships between the length of the interval preceding a clutch and the number, size or total mass of eggs produced (likelihood ratio test, clutch size: $\chi_1^2 = 0.13$, $p = 0.71$; egg mass: $\chi_1^2 = 0.017$, $p = 0.90$; total clutch mass: $\chi_1^2 = 0.07$, $p = 0.79$; $N = 36$ clutches from 15 females; **Figure 7.3**). Female size, measured as curved carapace length (CCL), was also unrelated to nesting interval length (multiple regression with SST as a covariate; $F_{1,44} = 0.04$, $p = 0.85$).

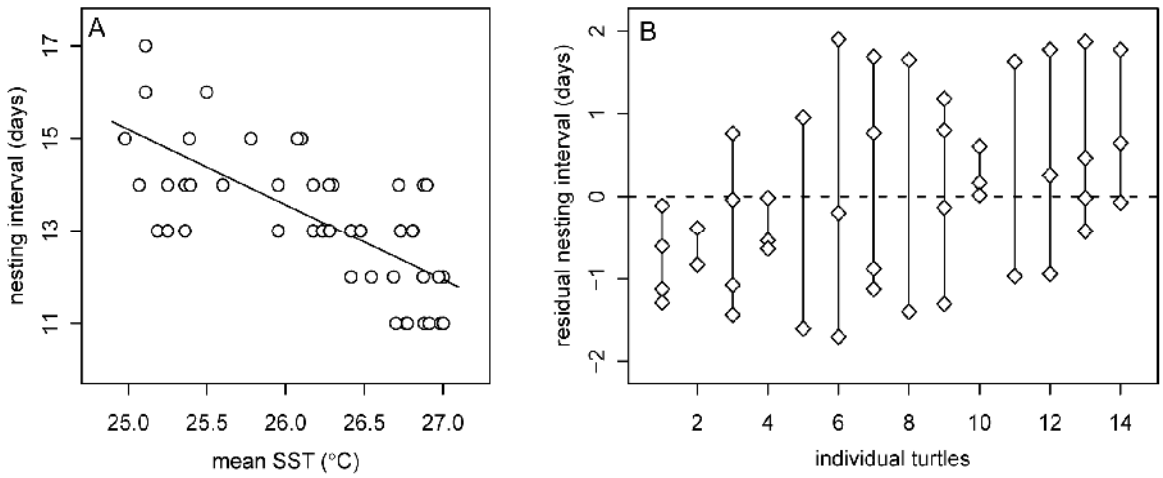



**Figure 7.2.** Effects of temperature and maternal phenotype on nesting interval length for green turtles nesting at Ascension Island. (A) Relationship between mean sea surface temperature (SST) during the nesting interval and interval length. (B) Repeatability of nesting interval lengths for individual turtles measured 2 or more times during the season ($N = 14$) after controlling for the effects of SST. Positive values indicate longer nesting interval lengths than predicted by the relationship with water temperature.

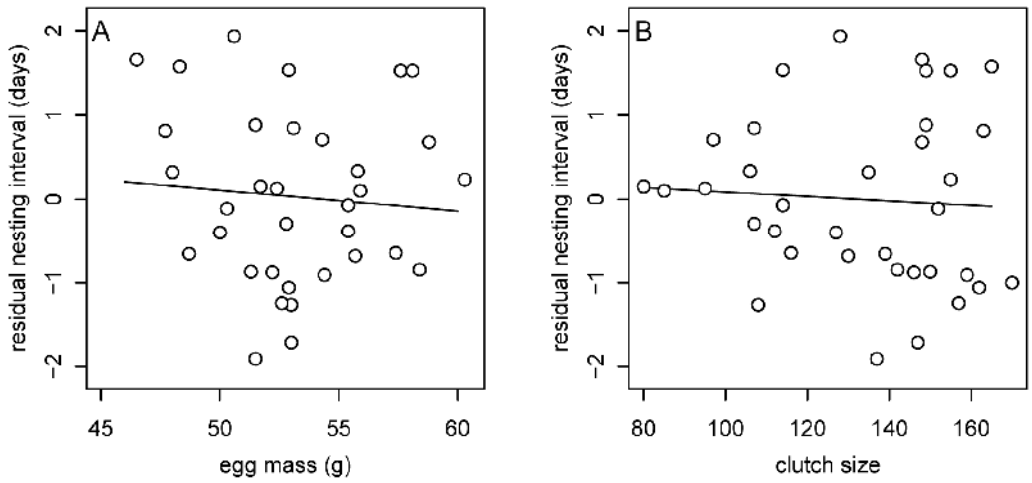



**Figure 7.3.** Effect of maternal reproductive investment on nesting interval length for green turtles at Ascension Island. Relationships between (A) egg size and (B) clutch size and nesting interval length after controlling for the effects of SST.

Thus, SST was the only significant determinant of nesting interval length for green turtles at Ascension Island. Previous studies using animal-mounted temperature loggers have directly estimated the relationship between water temperature and nesting interval length in sea turtles to fit the linear function log(length) = 2.25 – 0.043 × temperature (Hays et al. 2002a). For comparison, the regression equation linking log-transformed nesting interval length and remotely sensed SST in the present study was log(length) = 2.29 – 0.045 × SST ($r^2$ = 0.50), which did not differ significantly from the slope and intercept estimated by Hays et al. (*F*-test, $F_{2,44}$ = 1.2, $p$ = 0.30). This not only suggests that SST was a reliable measure of the actual water temperature experienced by females, but also implies that a single thermodynamic relationship may relate temperature and nesting interval length across different populations and species of marine turtles.

### 7.4.2 Metabolic rate and egg production

The metabolic theory of ecology (MTE; Brown et al. 2004) predicts that metabolic rate, and thus many other biological rates, will vary with body mass and temperature according to the fundamental equation:

$$\text{rate} = b_0 M^{3/4} e^{-E/kT} \qquad [1]$$

Where $b_0$ is a normalisation constant, $e^{-E/kT}$ is the Boltzmann-Arrhenius factor, which describes the temperature dependence of the rate (see below), and $M^{3/4}$ is an allometric scaling of body mass to account for the fractal nature of resource distribution networks in animals and the effects this has on metabolic rate over orders of magnitude of body size (Gillooly et al. 2001). This latter parameter is often less important in intraspecific comparisons as the range in body size tends to be relatively small (e.g. Tilman et al. 2004; Munch & Salinas 2009). In our study population female CCL ranged from 107 – 123 cm (equivalent to a range in body mass of ~140 – 200 kg; Hays et al. 2002b) and was unrelated to nesting interval length (see § 7.4.1). Previous studies similarly found no correlation between body mass and nesting interval length in marine turtles (Sato et al. 1998). Thus, the temperature-dependence of nesting interval length does not appear to be conditional on maternal body mass. Rearranging equation [1] to include only temperature effects gives the following:

$$\ln(\text{rate}) = \ln(b_0) - E(1/kT) \qquad [2]$$

Thus, according to MTE, log-transformed rate should vary as a linear function of $1/kT$, where $k$ is the Boltzmann constant and $T$ is absolute temperature (ºK), with a slope of $E$ which is the activation energy of the process. Moreover, for biological rates $E$ is predicted to fall in the range of activation energies for enzyme catalysed metabolic reactions (i.e. $E$ = 0.6 – 0.7 eV; Gillooly et al. 2001).

To test whether this prediction is upheld for the rate egg production in sea turtles I compiled a dataset of published nesting interval lengths and water temperatures for green and loggerhead turtles nesting in Japan ($N$ = 24; Sato et al. 1998), Cyprus ($N$ = 10; Hays et al. 2002a) and Ascension Island ($N$ = 48; this study; Hays et al. 2002a). Nesting interval length was converted to a rate of egg production by taking the reciprocal ($R$ = length in days$^{-1}$) and modelled as a linear function of $1/kT$ following equation [2]. The model explained a majority of the variation in the rate of egg production across populations (linear regression, $F_{1,80}$ = 226.7, $p < 0.001$, $r^2$ = 0.75; **Figure 7.4**) and was described by the linear function $\ln(R) = 27.7 - 0.78(1/kT)$. Residual deviances from the fitted model were not significantly different for nesting interval data taken from different populations (one-way ANOVA, $F_{2,79}$ = 0.035, $p$ = 0.97), indicating that the single model fits all nesting interval length data equally.

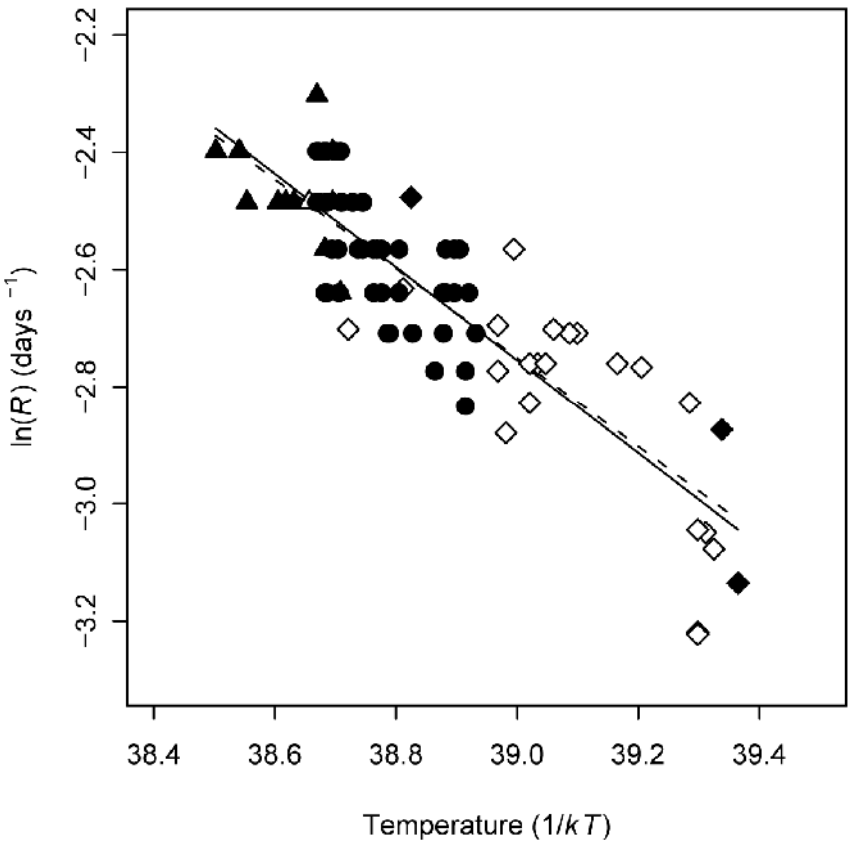



**Figure 7.4.** The relationship between ambient water temperature (1/*kT*) and the rate of egg production (*R* = nesting interval length$^{-1}$) for green turtles (solid symbols) and loggerhead turtles (open symbols) nesting in Cyprus (triangles), Ascension Island (circles) and Japan (diamonds). Data are plotted according to the Boltzmann-Arrhenius relationship (see equation [2]) where *k* is Boltzmann's constant and *T* is water temperature in ºK. The slope of the regression line fitted to these data (solid line, $y = 27.7 - 0.78x$) estimates the activation energy (*E*), or temperature dependence, for the rate of egg production as $E = 0.78$ eV. For comparison, the broken line shows a linear regression fit to these same data with slope constrained to $E = 0.76$ eV which is the estimated temperature dependence for mass-normalised metabolic rate in reptiles (Gillooly et al. 2001).

The slope of the model estimates the activation energy (*E*), or temperature dependence, for the rate of egg production in sea turtles as $E = 0.78$ eV. This is slightly outside the range of activation energies of 0.6 - 0.7 eV predicted for biological rates by MTE (Gillooly et al. 2001; Brown et al. 2004), although was not significantly different than a slope of $E = 0.7$ eV at the upper bound of this range (*F*-test, $F_{1,80} = 2.486$, $p = 0.12$). Moreover, the range predicted by MTE is based on the average activation energy across all taxa, whereas subtle taxonomic differences in the temperature dependence of

metabolic rate mean that taxon-specific estimates of $E$ may be more appropriate as a basis for comparison (Gillooly et al. 2001; Gillooly et al. 2006). Indeed, the activation energy for mass-normalised metabolic rate in reptiles specifically has been estimated as $E$ = 0.76 eV (extracted from Figure 1F in Gillooly et al. 2001), which is remarkably consistent with $E$ = 0.78eV estimated herein for the rate of egg production in marine turtles ($F$-test, $F_{1,80}$ = 0.178, $p$ = 0.67; **Figure 7.4**).

## 7.5 Discussion

### 7.5.1 Metabolic effects on egg production

The factors which determine how quickly females can produce eggs have been little studied in oviparous vertebrates, possibly reflecting technical difficulties associated with the measurement of this rate. In this respect marine turtles offer a rare opportunity, as the length of the interval separating successive clutches of individual females can be used as an inverse measure of the rate at which eggs mature. In this study we have shown that the rate of egg production in marine turtles varies as a function of ambient temperature in a manner that is remarkably consistent with the universal temperature dependence (UTD) predicted by the metabolic theory of ecology (MTE; Gillooly et al. 2001; Brown et al. 2004), but is largely independent of maternal phenotype and reproductive investment.

According to the MTE, the Arrhenius temperature-dependence of biological processes should consistently fall in the range $E$ = 0.6 – 0.7 eV which encompasses the average activation energies of enzyme-catalysed metabolic reactions (Gillooly et al. 2001; Brown et al. 2004). The slope of 0.78 estimated herein for the rate of egg production was not significantly different from the upper bound of the MTE predicted range, and closely approximated the slope of 0.76 estimated for metabolic rate in reptiles (Gillooly et al. 2001; **Figure 7.4**). This clearly implies that the thermodynamics of metabolism is rate-limiting for egg production. The observation that temperature affects the rate of egg maturation in oviparous animals and that this relationship probably has a metabolic basis is by no means new (Caroll & Quirting 1993; Calbet & Agusti 1999; Hays et al. 2002b), however the results of this study integrate egg production into the wider context of metabolic theory and provide further support for a UTD for biological rates. Indeed, in **Chapter 5** I show that the developmental times of green turtle embryos also vary

with temperature according to the UTD predicted by MTE ($E$ = 0.66 eV; extracted from **Figure A5.6.**, pg. 72), suggesting that this single temperature-rate relationship may underpin many aspects of marine turtle life-history.

Despite close agreement with the temperature-dependence predicted by MTE, there was nonetheless considerable residual variation in egg production rates that could not be explained by either water temperature or phenotypic differences among females. Nesting interval lengths were not dependent on the size, number or total mass of eggs produced in a clutch (**Figure 7.3**; see also Sato et al. 1998) and had low overall repeatability for individual females measured multiple times over the course of the nesting season (**Figure 7.2B**). Female body size was also unrelated to nesting interval length, which supports the finding that body mass generally has low predictive power for metabolic processes in intraspecific comparisons (Tilman et al. 2004; Munch & Salinas 2009). Given the small amount of variance explained by maternal phenotypes, it is possible that much of the residual variation in nesting interval lengths may be due to stochastic events that interrupt nesting patterns (e.g. rough seas, human disturbance), or an artefact of the integer scale on which nesting interval length is typically measured. Because most species of sea turtle nest nocturnally, nesting can only occur on one night or the next and therefore intervals cannot be measured on a truly continuous timescale, which will introduce error. Indeed, for the combined dataset the mean (± SE) residual deviation in interval length from the fitted relationship with water temperature was 1.0 ± 0.1 days (**Figure 7.4**), indicating that females typically nested on the night before or night after the exact time predicted by body temperature.

Given the physiologically integrated nature of egg production, it is perhaps unsurprising that the rate at which eggs are formed is dependent on metabolic processes. However, based on current understanding of the reproductive cycle of marine turtles it may be possible to comment further on the rate-limiting step(s). According to Owens (1980) and Licht (1982), ovulation of the full complement of yolky follicles for a clutch occurs soon after the previous clutch is laid, and is followed by an extremely rapid phase of albumen deposition in the oviducts that is typically complete within 72 hours. Thus, formation of the major constituent parts of eggs is unlikely to limit the rate of egg production, which is consistent with the finding that neither the size nor number of eggs in a clutch contributes to nesting interval length. In contrast, shell membrane formation and calcification in the lower oviduct occurs more slowly and may therefore limit how

quickly eggs can be produced (Owens 1980; Hamann et al. 2003). Egg shell mineralisation is an active process involving ATP-dependent transport of calcium ions across the uterine epithelium (Thompson et al. 2007) and might therefore be limited by metabolic rate. Alternatively, embryonic development whilst still in the oviducts might limit the rate at which mature eggs can be produced, as in many snakes and lizards (e.g. Lourdais et al. 2008). This seems unlikely in sea turtles as embryonic development is arrested at a very early stage until shortly after the eggs are laid (Miller 1985). Nonetheless, developmental rate is known to vary with temperature according to the predictions of MTE in various species (Gillooly et al. 2002), including sea turtles (**Chapter 5**, pg. 71), and may therefore contribute to the overall rate of egg production.

In conclusion, this study has shown that the rate of egg production in marine turtles varies with ambient temperature in accordance with the predictions of MTE, but is largely independent of maternal phenotype and reproductive investment. Because operating temperature and its effects on metabolism apparently underpin much of the variation in fitness-related biological rates, there is considerable scope for selection to act on this property of organisms and shape it adaptively (Brown et al. 2004). Indeed, in the next section I review evidence that the relationship between temperature, metabolic rate and egg production has had important consequences for maternal thermoregulatory behaviour in marine turtles.

### 7.5.2 Implications for female behaviour

Egg production is costly for females, diverting time and resources away from other life-history processes. In addition to the direct energetic and nutritional investment in eggs, females may also incur costs through the physiological and behavioural changes that accompany reproduction e.g. altered hormonal states and haematology, reduced foraging opportunities and food intake, and increased predation risks associated with carrying eggs (Shine 1980; Lourdais et al. 2002; Williams 2005). Many of these latter costs are likely to be conditional on the duration of the reproductive period and may therefore confer a selective advantage to females that can produce eggs quickly.

Given that the rate of egg production in marine turtles is largely determined by ambient temperature and is influenced little by maternal phenotypes, if rapid egg production is

adaptive we may expect breeding females to facultatively increase their operating temperatures through behavioural thermoregulation. Indeed, a recent study by Schofield et al. (2009) has provided the first explicit evidence that gravid loggerhead turtles (*Caretta caretta*) maintain higher body temperatures than would be expected from ambient conditions by actively tracking warm water microhabitats. They suggest that this behaviour may serve to accelerate egg production in a population at the latitudinal extreme of the species' range, where the window of suitable climatic conditions for incubating eggs is correspondingly short.

However, the fact that thermophilic behaviour has only recently been recognised in breeding marine turtles may reflect the technical difficulties associated with monitoring behaviour in these species, rather than it being a localised phenomenon. Indeed, evidence from globally distributed populations suggests that the need to maximise operating temperatures has a profound effect on female habitat utilisation during egg production. For example, loggerhead turtles nesting in Japan have been shown to travel considerable distances during the inter-nesting period, actively seeking out warm water currents (Naito et al 1990; Sakamoto et al. 1993), which suggests that thermoregulatory requirements may sometimes outweigh the energetic costs associated with such migrations. Leatherback turtles (*Dermochelys coriacea*) nesting in French Guiana also range widely between nesting events, yet similarly restrict their movements to the warmest areas of the inter-nesting habitat (Fossette et al. 2009).

In contrast, at many breeding sites females are relatively quiescent during the inter-nesting period, preferring to rest in shallow coastal waters to conserve energy (e.g. Hays et al. 2000; Houghton et al. 2002). Nonetheless, significant opportunities for thermoregulation may still arise from selection of vertical position in the water column. Water temperatures decrease from the ocean surface to greater depths, but the degree of thermal stratification varies among breeding sites and depth selection by gravid females appears to track this variation. For example, in Mediterranean rookeries such as Cyprus and Zakynthos, where sheltered conditions and calm waters lead to the formation of a relatively shallow surface mixed layer (with mean water temperature dropping by 1-2 $^{o}$C between the surface and a depth of 5-10 m; Hays et al. 2002b; Schofield et al 2009), female green and loggerhead turtles almost exclusively select resting depths shallower than 4-6 m (Hays et al. 2002d; Schofield et al 2009; Fuller et al 2009). By contrast at exposed oceanic nesting sites like Ascension Is., where the warm surface mixed layer

extends deeper, with little variation in mean water temperature from the surface to 20 m (Hays et al. 2002b), female green turtles select resting depths between 12 - 18 m (Hays et al. 2000; Hays et al 2002d).

Deeper resting depths are thought to be advantageous for gravid turtles, allowing them to maintain neutral buoyancy whilst holding greater amounts of air in the lungs, thus minimising the energy expended on resurfacing to breathe (Hays et al. 2000). However, the available evidence suggests that females must balance this against the need to remain in the warm surface layer and maximise body temperature. Indeed, while females nesting at Ascension Is. select deeper resting depths than those in the Mediterranean, behaviour at this rookery varies diurnally, with females spending less time resting and moving to depths shallower than 5 m in the middle of the day when transient warm surface layers may form (Hays et al 2000; Hays et al 2002d), consistent with active thermoregulation. In some tropical breeding grounds where nesting occurs year round, females also appear to alter their depth selection in response to seasonal changes in water column stratification in order to stay within the warm surface mixed layer (Yasuda et al. 2008). At a small number of nesting sites females may even leave the water column altogether and bask on land (summarised in Whittow & Balazs 1982). This behaviour is relatively rare and its biological function is not well understood, however terrestrial basking significantly elevates female body temperatures and it has been suggested that it may serve to accelerate egg production (Whittow & Balazs 1982).

Overall, while explicit behavioural studies are currently limited, there is compelling evidence to suggest that gravid marine turtles actively increase their body temperatures through behavioural thermoregulation and microhabitat selection. This finding may be unsurprising given the prevalence of ‘maternal thermophily’ in terrestrial reptiles, particularly amongst the squamata. Gravid lizards and snakes are frequently observed to bask more and maintain higher body temperatures compared to conspecifics (e.g. Graves & Duvall 1993; Blazquez 1995; Blouin-Demers & Weatherhead 2001; Shine 2006; Lourdais et al. 2008). However the adaptive significance of maternal thermophily in squamates is unclear. While such behaviour can significantly increase the rate at which eggs mature, and so reduce length of time they must be carried by females prior to oviposition (Lourdais et al. 2008), squamates also have prolonged retention of eggs *in utero* (with as much as one-third of embryonic development occurring prior to oviposition; Andrews 2004) meaning this behaviour may equally serve to optimise the

developmental environment for offspring (Wapstra 2000; Shine 2006). In contrast, chelonids lay their eggs at a very early stage in development (when the embryo is not yet visible; Miller 1985) suggesting there is little scope for maternal thermoregulatory behaviour to influence offspring phenotypes. Thus, maternal thermophily in marine turtles provides less ambiguous evidence that females may adjust their body temperature in order to increase metabolic rate and accelerate egg development. The benefits may be profound: based on the relationship estimated in this study (**Figure 7.4**), for a female laying 4 clutches during the nesting season and experiencing an ambient water temperature of 25 ºC, maintaining a body temperature 2 ºC higher than ambient through behavioural thermoregulation can reduce the total time invested in egg production by 11 days (a reduction of ~ 20 %).

# **Chapter 8** General Discussion

The publication of a landmark volume by Mousseau & Fox (1998a) focussed interest on maternal effects as adaptive strategies for coping with environmental heterogeneity: a principle which has guided much of the subsequent research in the field (Marshall & Uller 2007). This thesis has examined two specific maternal traits in the green turtle (*Chelonia mydas*), namely the provisioning of fat-soluble antioxidants in eggs (**Chapters 2-5**) and the selection of oviposition sites via natal homing (**Chapter 6**). In the following pages I summarise the key findings of this work with a view to establishing the adaptive significance of these maternal traits. In **Section 8.1** I suggest that while maternally-derived antioxidants in eggs may have a role and a requirement during early development, much of the variation in provisioning among and within the clutches of individual females stems from non-adaptive physiological constraints and may be of little functional relevance for offspring. In **Section 8.2** I propose that natal homing may significantly enhance parental/offspring fitness by maintaining inter-generationally stable environments against which other key fitness traits can evolve, and discuss the implications of this finding for the evolution of homing behaviour itself.

## 8.1 Fat-soluble antioxidant provisioning in eggs: adaptive maternal effect or physiological inevitability?

Carotenoids and vitamin E are ubiquitous constituents in the eggs of birds, reptiles and many fish, where they have been suggested to function as antioxidants that protect developing embryos and neonates from free-radical induced oxidative stress (Surai & Speake 1998; Blount et al. 2000; Surai 2002; Biard et al. 2005; McGraw et al. 2005; **Chapter 1**). However, a number of recent studies have cast doubts on the ability of carotenoids to mitigate oxidative stress (Constantini & Møller 2008; Isaksson & Andersson 2008; Olsson et al. 2008; **Chapter 5**) and speculated that they may be passively incorporated into the lipid matrix of egg yolk as an inevitable consequence of their solubility in fats (Karadas et al. 2005; Badyaev et al. 2006; Grether et al. 2008; **Chapter 5**). So is there an adaptive role for maternally-derived antioxidants in eggs? Here I address two important questions relevant to this debate: what are the proximate mechanisms underlying variation in maternal antioxidant provisioning to eggs and what (if any) are the functional consequences of this variation for offspring?

Over the past decade a large number of studies have tested the effects of yolk-derived carotenoids on offspring growth, immunity and survival and produced inconsistent results, sometimes in the same species (**Table 1.1.**). Where detected, the beneficial effects of yolk carotenoids for offspring are often attributed to their antioxidant properties (e.g. Biard et al. 2005; McGraw et al. 2005); a claim which is at odds with much recent evidence that carotenoids are minor antioxidants under physiological conditions (Constantini & Møller 2008; Isaksson & Andersson 2008; Olsson et al. 2008). In **Chapter 5** I explicitly tested the antioxidant function of yolk-derived carotenoids and vitamin E in hatchling green turtles and found no capacity of either compound to suppress oxidative stress over a 3-4 fold range in yolk concentrations, despite statistically controlling for many important aspects of the developmental environment. The results for vitamin E are surprising given its established role as an antioxidant in animals (Traber & Atkinson 2007), although several studies in humans have suggested that the importance of vitamin E may have been overstated, with noticeable reductions in oxidative damage requiring pharmacological doses as high as 30 times the RDA for this vitamin (Meagher et al. 2001; Roberts et al. 2007).

Since no other studies have investigated the effects of yolk antioxidants on offspring resistance to oxidative stress *in vivo* it is hard to draw general conclusions from these results. For example, it is possible that female sea turtles do not provision their eggs with sufficient antioxidants to mitigate oxidative stress to any great extent because high extrinsic mortality risk puts offspring survival beyond maternal control (see Frazer 1986). It will therefore be interesting to see if the higher levels of antioxidants typically found in avian eggs confer protection against oxidative stress (e.g. **Figure 2.3**). However, it is equally possible that the multifaceted and highly integrated nature of the vertebrate antioxidant system creates an effective buffer against variations in the level of any one component (Halliwell & Gutteridge 2007; Monaghan et al. 2009). Endogenous antioxidant defences in the embryo and neonate may therefore easily compensate for low levels of maternally-derived antioxidants. This would explain the disparity between *in vitro* studies which found a protective effect of yolk-derived vitamin E and carotenoids on embryonic tissues against an oxidative challenge (Surai & Speake 1998; Surai et al. 1999), and our *in vivo* study which found no such effects. Interestingly, mammalian oocytes contain significant quantities of maternally derived mRNA transcripts for powerful antioxidant enzymes such as superoxide dismutase (Guérin et al 2001), suggesting that mothers may give the offspring antioxidant system

a headstart in ways other than through diet-derived antioxidants. Exogenous antioxidants may therefore be particularly important during very early developmental stages before the embryo begins to express its own defences, which would explain the known requirement for some maternally-derived vitamin E in order to prevent early embryo death (Debier & Larondelle 2005). Nonetheless, the lack of relationship between yolk vitamin E concentrations and hatching success in green turtles suggests that the amount required may be low and invariably met by females (**Chapter 5**; but see Møller et al. 2008). Establishing where such thresholds lie through experimental reductions in yolk vitamin E would be relatively straightforward and could establish whether maternal vitamin E provisioning is ever likely to compromise embryo survival.

In contrast to vitamin E, carotenoids do not appear to be essential for normal embryonic development. Early work in poultry and fish showed that hatchlings from eggs completely devoid of carotenoids developed, hatched and matured normally (reviewed in Goodwin 1950). Equally, yolk carotenoids are clearly not functionally inert given the number of studies that have reported effects on offspring body size and indices of immune function (**Chapter 5**; **Table 1.1**). Our results suggest that such effects are not mediated by any antioxidant role (see also Saino et al. 2008; Perez-Rodriguez 2009), although they may still be related through the effects of carotenoids on cell proliferation and differentiation, which could influence both growth and immunity (Stahl et al. 2002; Sharoni et al. 2004). However, while interesting, these findings are not sufficient to conclude that carotenoid deposition in eggs enhances maternal and/or offspring fitness. Modifications to offspring body size are typically marginal (Biard et al. 2005; Ewen et al. 2008; Saino et al. 2008; **Chapter 5**), and recent reviews have cautioned against using simple immune indices such as those normally related to yolk-derived carotenoids (e.g. the PHA response) in order to infer immunocompetence and survival prospects (Kennedy & Nager 2006; Owen & Clayton 2007). Indeed, studies that have related yolk carotenoid levels to utlimate measures of fitness (i.e. survival of embryos and hatchlings) have almost invariably proven negative (**Table 1.1; Chapter 5**). This highlights a clear need for more long term studies of the fitness consequences of variable carotenoid (and vitamin E) deposition in eggs.

Given the lack of convincing evidence for effects on offspring fitness, it may be helpful to ask why yolk concentrations of carotenoids and vitamin E vary so dramatically among females in most wild populations (by as much as 30-fold; Biard et al. 2005;

Isaakson et al. 2008; **Chapters 4 & 5**): does this reflect active adjustments, resolutions to trade-offs or idiosyncrasies of individuals? Experimental and comparative studies have repeatedly shown that concentrations of carotenoids and vitamin E in egg yolk are influenced by their availability in the mothers' diet (Blount et al. 2002a; Grobas et al. 2002; Royle et al. 2003; Cassey et al. 2005; **Chapter 3**), which has been taken as evidence of maternal limitation (e.g. Biard et al. 2005; de Neve et al. 2008). However, such a relationship could equally arise from passive diffusion, as illustrated by the efficient transfer of fat-soluble dyes from the diet into egg yolk (Astheimer et al. 1989). In fact, the hypothesis that dietary access to antioxidants varies within populations is virtually untested and has been strongly questioned given the abundance of these molecules in most marine and terrestrial food webs (Hudon 1994); a contention supported by the similar degree of variation in yolk antioxidant levels among wild green turtles compared to captive turtles fed on a uniform diet (**Chapter 3**). Studies in wild birds have likewise shown that individual variation in carotenoid deposition in plumage is not diminished when animals are placed on a uniform and/or highly supplemented diet (McGraw & Hill 2001; McGraw et al. 2003b; Hadfield & Owens 2006). The implication from these studies is that post-ingestion, physiological mechanisms underpin most of the variation in antioxidant availability and utilisation within populations.

An interesting finding arising from this thesis is that different physiological pathways appear to underpin variation in the deposition of vitamin E and carotenoids in eggs. Carotenoid concentrations in yolk decreased across successive clutches of individual green turtles and were strongly correlated with concentrations in maternal blood plasma, whilst yolk concentrations of vitamin E increased across successive clutches and were unrelated to blood levels (**Chapters 3 & 4**). A similar dichotomy also seems to exist in other taxa. In zebra finches the size of maternal adipose reserves was shown to predict carotenoid levels but not vitamin E levels in eggs (Williamson et al. 2006), and in humans, carotenoid concentrations in breast milk were found to correlate with maternal plasma levels whilst vitamin E concentrations were not (de Azeredo & Trugo 2008 and refs therein), thus mirroring the results of our study of green turtle eggs (**Chapter 4**).

The available evidence suggests that carotenoid concentrations in eggs may be largely constrained by the size of endogenous reserves in the mother. Firstly, in sea turtles and most birds, carotenoid concentrations in the eggs of individual females progressively

decline across the reproductive effort (**Chapter 3**; Royle et al. 1999; Blount et al. 2002a). This trend is observed across successive eggs in birds and across successive clutches in turtles, which is intuitive given that reptilian clutches consist of populations of follicles that mature simultaneously, and thus experience the same maternal physiochemical environment (Callard et al. 1978). Whilst some studies have proposed an adaptive role for within-female variation in carotenoid provisioning (Royle et al. 1999, 2001; Horak 2002), the fact that systematic reductions in egg levels are found in both birds and turtles with very different reproductive strategies tends to suggest that it is a universal constraint of egg production linked to the depletion of maternal reserves (see also Badyaev et al. 2006; Groothuis et al. 2006). A link between maternal carotenoid reserves and carotenoid deposition in eggs is further supported by strong correlations between yolk concentrations and maternal plasma concentrations in various taxa (**Chapter 4**; Bortolotti et al. 2003; McGraw et al 2005; Isaakson et al. 2006, 2008), since plasma carotenoid levels tend to be indicative of quantities stored in other tissues (e.g. adipose, liver and integument; El Sohemy et al. 2006; McGraw et al. 2003; McGraw & Toomey 2010). Given that diet appears to be a relatively minor source of variation in carotenoid status within wild populations (see above), much of the variation in maternal reserves presumably arises from post-ingestion processes. Studies in birds and humans have indeed revealed marked individual variation in the ability to absorb and utilise dietary carotenoids amongst healthy subjects under controlled conditions (Dimitrov et al. 1988; McGraw et al. 2003; Karu et al. 2007; Perez-Rodriguez 2008), which is hard to reconcile with the purported essential functions of carotenoids.

In contrast to carotenoids, yolk vitamin E concentrations increased across successive clutches of individual green turtles, which might imply active regulation. In **Chapters 3 & 4** I propose a possible hormonal mechanism to explain variable vitamin E deposition in eggs, based on the observation that oestrogen (and also testosterone) stimulates increased circulating levels of vitamin E in female birds (Halifeoglu et al. 2003). Such a mechanism might explain the different within-female patterns of vitamin E deposition in turtles, where maternal plasma oestrogen and yolk vitamin E levels increase across the reproductive effort (Rostal et al. 1998; **Chapter 3**), and birds where yolk levels of vitamin E and plasma oestrogen typically decline over the laying sequence (e.g. Williams et al. 2004; Williamson et al. 2006). The role of reproductive hormones in coordinating other aspects of egg composition is an interesting line of further work and warrants experimental testing using exogenous hormones. However, there is little

evidence currently to suggest that females adaptively adjust vitamin E levels in eggs to specific environmental conditions. The increase in vitamin E deposition in eggs that we found across successive clutches of green turtles might be an adaptive response to seasonally increasing temperatures at Ascension Island (**Figure 7.1**). Such chnages can affect offspring development rates (**Figure A5.2**) and possibly the risk of oxidative stress (e.g. Nussey et al. 2009; Hall et al. 2010). However, we found no evidence that development rates affect oxidative stress risk in hatchlings, that vitamin E levels in eggs influence oxidative stress risk, or that females tailor antioxidant concentrations in eggs to specific nest environments, either within beaches (**Chapter 5**), or among beaches with broadly different thermal regimes (**Chapter 6; Table A6.1**). This raises the possibility that 'active' control of vitamin E deposition may in fact be a by-product of other physiological processes, such as changes in maternal plasma oestrogen levels associated with follicular growth and ovulation patterns (**Chapter 3**)

In conclusion, the observation that female reptiles and birds readily transfer lipid soluble synthetic dyes, organic pesticides and other environmental pollutants into their eggs suggests that the non-specific deposition of fat-soluble metabolites into eggs may be inevitable (Astheimer et al. 1989; McKenzie et al. 1999; de Solla et al. 2001). This places an onus on researchers to demonstrate that fat-soluble antioxidant provisioning in eggs is adaptively adjusted or limiting for offspring quality. I would suggest that currently there is insufficient evidence to conclude that variation in maternal vitamin E and carotenoid deposition in eggs is limited by dietary access, tailored to specific environments, or is an important determinant of offspring fitness and/or oxidative stress resistance. Some degree of antioxidant protection from maternally-derived vitamin E is clearly necessary for development to proceed, and yolk carotenoids may have a specific antioxidant role in offspring visual function, which remains little explored (e.g. Krinsky et al. 2003). However it seems plausible that mothers invariably meet such requirements through the passive transfer of fat-soluble antioxidants in yolk lipoproteins. To definitively test whether yolk-derived vitamin E and carotenoids have a role in buffering offspring from oxidative stress it may be helpful to experimentally reduce concentrations in eggs, and/or chemically induce oxidative stress in offspring (e.g. Isaksson & Andersson 2008) in order to account for the possibility that most females meet the antioxidant needs of their offspring in wild populations. There is also a clear requirement for long-term studies linking antioxidant levels in eggs to the lifetime fitness of offspring, if tractable study systems can be found.

## 8.2. Natal homing is an adaptive maternal effect driving evolutionary change

Maternal effects, once viewed as 'nuisance parameters' in quantitative genetic studies (Falconer 1981), are now widely recognised as agents of evolutionary change (Wade 1998; Wolf & Brodie 1998; Räsänen & Kruuk 2007). A particular example of this relates to oviposition behaviour in oviparous animals. In such species, the maternal genotype not only contributes directly to offspring genotypes, but may also determine the environment in which offspring genes are expressed through oviposition site choice (Mousseau & Fox 1998b). Thus, offspring genes may adapt to perform optimally in the maternally-provided environment and maternal oviposition preference genes may evolve in concert to maintain a consistent selective environment for offspring (Resetarits 1996; Wade 1998; Wolf & Brodie 1998; Wolf 2000; **Figure 8.1A**). In **Chapter 6** I present novel evidence that selection of nest sites via natal homing in female green turtles may similarly facilitate adaptation of their offspring to specific environmental regimes, thereby increasing maternal/offspring fitness. In the following pages I elaborate on these findings and discuss their implications for the evolution of homing behaviour in animals more generally.

Whilst not typically considered a maternal effect, natal homing dictates the environment in which offspring will develop and can therefore be viewed as a form of oviposition site choice (I restrict my discussion initially to homing in females and return to males later). Homing differs from 'classical' models of oviposition site selection (e.g. Wade 1998; Wolf 2000; **Figure 8.1A**) in that maternal oviposition preferences are inherited culturally (rather than genetically) by females imprinting on the location of their own natal sites (Quinn & Dittman 1990; Lohmann et al. 2008). Thus, a single maternal 'homing' genotype can produce multiple offspring environments (or 'envirotypes'; *sensu* Mameli 2004) depending on where a female herself was born. However, with this caveat in mind, natal homing may have a similar adaptive basis to genetically determined oviposition preferences (**Figure 8.1B**).

Assuming that the natal environment remains stable over generational timescales (a big assumption, as discussed later), maternal selection of oviposition sites through homing may maintain a consistent environmental context to which offspring traits can adapt (Resetarits 1996). Until now, the clearest evidence for this has come from the salmonid fish. It has been known for over a century that breeding populations of salmon exhibit

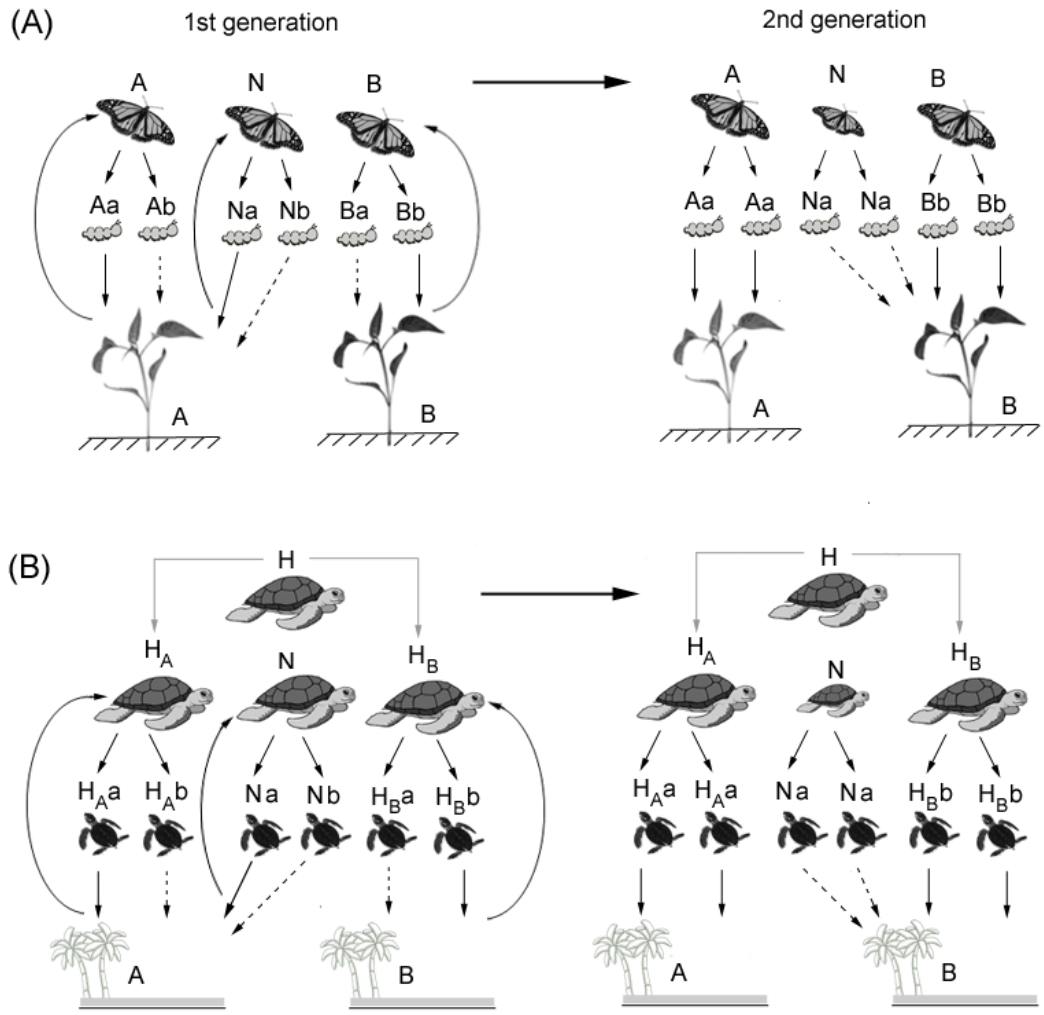



**Figure 8.1.** Conceptual diagrams illustrating the coevolution of maternal oviposition preferences and offspring adaptations via alternative mechanisms. **(A)** Coevolution of a genetically determined oviposition preference (e.g. choice of host plant; upper case letters) and a maternally-inherited offspring trait which determines survival on a specific host (e.g. an enzyme; lower case letters). Preference A chooses plant A, B chooses plant B and N expresses no preference. Only offspring with enzyme 'a' survive on plant A, and only enzyme 'b' on plant B, other combinations have zero fitness (broken arrows). In the first generation all maternal genotypes produce offspring with both enzymes and have equal fitness. All surviving offspring of A females inherit both preference A and enzyme 'a' which results in increased fitness in the second generation (and similarly for B females); however the surviving offspring of N females inherit no preference and continue to place many of their own offspring inappropriately. Thus 'choosy' maternal genotypes A and B ultimately have higher mean fitness and spread. **(B)** Evolution of natal homing through a similar coadaptive process, with the exception that a single maternal homing genotype (H) can produce multiple preferences ('envirotypes'; as subscripts) depending on where a female herself was born which are then culturally inherited by offspring through imprinting. Each envirotype may become coupled with offspring viability traits that increase fitness in its natal environment with the overall effect that the H genotype spreads.

'local peculiarities' and specialisations to their natal spawning grounds (reviewed in Taylor 1991; Quinn 2005). These include locally adapted migratory behaviour (Raleigh 1971), juvenile morphology (Taylor 1991) and thermal tolerances of embryos and larvae (Beacham & Murray 1988; Taylor 1991; Hendry et al. 1998). In **Chapter 6** I suggest that fine-scale homing in sea turtles may similarly lead to adaptive divergence in embryonic thermal tolerances to suit the natal environment. In fact whilst explicit evidence is lacking for other species, local adaptation may prove to be a ubiquitous feature of natal homing. For example, it is known that many species of amphibians migrate to their natal spawning sites to breed (Berven & Grudzien 1990; Reading et al. 1991; Gamble et al. 2007) and that their larvae often display a range of habitat-specific adaptations at microgeographic scales (including thermal tolerance: Skelly & Freidenberg 2000; acid tolerance: Räsänen et al. 2003; morphology: van Buskirk & McCollum 1999; and life history: Weitere et al. 2004); although these phenomena have never been explicitly linked in any species.

Thus, there is growing evidence (both direct and circumstantial) that natal homing can drive adaptive divergence in offspring traits in various animal taxa, raising interesting questions as to how local adaptation might shape the evolution of homing behaviour itself. Evolutionary explanations for homing in sea turtles and various other taxa have tended to focus on the general assurances of breeding habitat quality that individuals derive from breeding at their own natal site (the 'failsafe hypothesis'; Brown & Shine 2007; Lohmann et al. 2008). Given the apparent prevalence of habitat-specific adaptations among homing species, a possible refinement of this hypothesis is that homing evolves because it ensures individuals breed in an environment that is compatible with the *specific genes* that their offspring will inherit (**Figure 8.1B**). Individuals breeding at sites to which they and/or their offspring are better adapted will leave more surviving offspring, which will in turn inherit the homing tendency, the particular envirotype and the genes adapted to it. This could be viewed as a coadaptive process similar to that proposed for genetically determined oviposition preferences (Wade 1998; Wolf 2000), wherein each envirotype becomes coupled with specific adaptations that increase fitness at its natal site; the overall effect that the homing genotype becomes more common (**Figure 8.1B**).

Whether such a process could be initiated without a tendency for homing already established, or whether it may simply act as positive feedback is difficult to say.

However, a level of local adaptation may be inherent within emergent natal homing systems from the outset due to the 'favoured founders effect' (Quinn et al. 2001). That is, in the first generation to breed at a particular site, those individuals which successfully produce viable offspring (the founders) will be by definition the ones that are best adapted to the prevailing environmental conditions, thus conferring an immediate fitness advantage to any of their offspring returning to breed at this site as adults.

A coadaptive explanation for natal homing generates several predictions which might be amenable to comparative testing. Firstly, if homing coevolves with habitat-specific adaptations we might expect such behaviour to be most highly developed in spatially heterogeneous and temporally stable environments that favour local adaptation (Kawecki & Ebert 2004). The precision of homing is known to vary considerably among different populations and species of marine turtles and salmon, ranging from crude regional philopatry, to exceptionally fine scale fidelity to individual beaches or gravel patches (Quinn 2005; Bowen & Karl 2007). However, few studies have related such differences to habitat structure (see Neville et al. 2006 for an exception in salmon).

Depending on the mode of inheritance of local adaptations, the intensity of selection on homing behaviour may also vary between the sexes. For example, if the traits which determine offspring fitness in the natal environment are maternally inherited we might expect homing to be most developed in females, as appears to be the case in various animal species (Neville et al. 2006; Bowen & Karl 2007; Campbell et al. 2008; see also **Box 8.1**). The results of our study of green turtles (**Chapter 6**) suggest that offspring thermal tolerance may be one such trait which could evolve along maternal lineages via mitochondrial genes, and appears to be a common adaptation to natal sites in homing species (see above). In contrast, if the traits which determine fitness in the natal site are biparentally inherited, selection may favour assortative mating and homing in both males and females. One obvious trait which is likely to be important in both sexes is breeding phenology, since appropriate conditions for offspring development often vary seasonally and males and females must synchronise their arrival at the natal breeding grounds to locate mates. Phenology has a strong genetic component in many species (Räsänen & Kruuk 2007), and given that suitable times for breeding are likely to vary among sites, may be locally adapted to a specific context. For example, ambient temperature is an important determinant of offspring viability in ectotherms (**Chapter**

**6**; Birchard 2004) and both salmon and sea turtles time their arrival at their natal breeding grounds to coincide with optimal developmental temperatures (Taylor 1991; Webb & McLay 1996; Hirth 1997). Indeed, maintaining cross-generational environmental stability for the evolution of offspring adaptations may require that parents not only return to a specific location but also arrive at that site at a particular time. Thus homing may provide a stable context for the coevolution of suites of inter-related traits that include parental breeding phenology and offspring developmental tolerances.

In conclusion, I suggest that natal homing may be adaptive because it maintains a stable environmental context against which other key fitness traits can evolve, and that this may (at least partially) underpin the evolution of homing itself. In fact, the defining

**Box 8.1. Culturally-inherited oviposition preferences and coadaptation in cuckoos**

An avian analogy to natal homing in fish, reptiles and amphibians may be found in the common cuckoo (*Cuculus canorus*); a brood parasite in which individual females specialise in laying their eggs within the nests of a particular host species. Evidence suggests that maternal host preferences are not genetically determined (Brooke & Davies 1991) but are instead culturally inherited through females imprinting on their own natal habitat or foster parents (Teuschl et al. 1998; Vogl et al. 2002; Aviles & Møller 2004; but see Brooke & Davies 1991). This behaviour has caused the divergence of cuckoo populations into distinct races (or gentes) exhibiting host-specific adaptations: female cuckoos produce eggs that closely mimic the colour and pattern of the host species, thus reducing the risk of rejection by the foster parents (**Figure 8.2**; Brooke & Davies 1988), and their offspring show innate (non-learned) sensitivity to the alarm calls of the specific host (Davies et al. 2006). Interestingly, population genetics suggest that only females imprint on their foster parents and that host-specific adaptations have thus evolved along maternal lineages, with male-mediated gene flow maintaining the various gentes as a single interbreeding species (Gibbs et al. 2000).

**Figure 8.2** Egg mimicry in cuckoos. Left column shows host egg (top: meadow pipit; bottom: great reed warbler) and right column shows respective cuckoo egg.†

† *Adapted from Brooke & Davies (1988) with permission from MacMillan Publishing Ltd*

feature of natal homing – the cultural inheritance of oviposition environments via imprinting – may be shared by a broad range of conceptually similar oviposition strategies in animals which likewise facilitate adaptation to specific environments. For example, an interesting analogy to natal homing is found in brood parasitic birds, which often select nests to parasitize by imprinting on the species of their own foster parents, resulting in distinct host races each exhibiting host-specific adaptations (**Box 8.1**). Many animals also imprint on their natal habitat type (if not the exact location) in order to select nest or oviposition sites, which may generate and maintain local adaptations (reviewed in Davis & Stamps 2004). Even in phytophagous insects, which have provided some of the classic case studies of oviposition preference - offspring performance coevolution, females of certain species appear to select oviposition sites by imprinting on olfactory or gustatory characteristics of their natal host plant (the controversial Hopkins' host selection principle; Jeanike 1988; Rietdorf & Steidle 2002; Facknath & Wright 2007). Thus, in complex environments where the potential determinants of fitness are diverse or difficult to assess, imprinting (or 'homing') mechanisms of oviposition site choice may offer the most efficient evolutionary strategy for maintaining constant selective environments across generations.

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