Mothers Have Favourites

Egg Composition, Mate Attractiveness and Maternal Effects in the Zebra Finch

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Contents

Abstract

Maternal effects are a means by which mothers can alter offspring phenotype, potentially enhancing offspring survival and their own lifetime reproductive fitness. In birds, maternal effects include egg composition. Mothers are expected to make a greater investment in offspring with a higher reproductive value.

Previous research has shown that female zebra finches (*Taeniopygia guttata*) deposit more yolk androgens in eggs laid for attractive males (Gil *et al.* 1999). I investigated the consequences of potential changes in egg composition due to mate attractiveness on the development of zebra finch chicks. The results showed clear evidence of maternal effects on offspring development that were due to components of egg composition. Females mated to attractive males laid larger eggs, chicks hatching from these eggs begged more and showed greater than expected tarsus growth. For the offspring as adults, females with attractive fathers were larger and laid larger eggs. However, I was not able to attribute our findings to increased levels of yolk androgens.

Antioxidants are also an important component of egg composition. I examined the relationship between mate attractiveness and yolk antioxidant concentration. Females paired to attractive males laid clutches with higher antioxidant levels in later laid eggs.

Finally, the mechanism by which females can alter the levels of yolk androgens in the eggs they lay is unclear. Yolk androgens may be derived exclusively from the steroidogenic activity of the follicle cells or it is possible that androgens in blood plasma may be an additional source. I investigated the relationship between plasma and yolk androgens across the laying cycle.

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Introduction

The phenotype of an individual is determined through a combination of its genotype and the environment it experiences during development. It is recognized that parents are responsible for the genetic element in this relationship, but it is now apparent that they can also influence the environmental component. Through their behaviour or the resources they provide, parents can alter the environment their offspring experience during development and thereby influence its phenotype. Since either parent can influence development in this way, these effects are broadly termed 'parental effects'. However, because in many animal species the mother is more often responsible, they are commonly known as 'maternal effects'.

Maternal Effects

Maternal effects are widespread: Mousseau and Fox describe them as being 'ubiquitous in nature' (Mousseau and Fox 1998a). Maternal effects cover a wide variety of phenomenon and can occur at any of the life stages. The particular stage at which maternal effects are important varies depending on the life history strategy of the species. Maternal effects may occur at the pre-zygote stage, during egg production, where mothers may influence egg size, the number of eggs produced, offspring sex and egg composition in terms of the nutrients contained within the eggs. At the zygote stage, during embryonic development, mothers may alter egg incubation conditions or the intra-uterine environment. Finally, following birth or hatching at the post zygote stage maternal care in terms of lactation, nestling provisioning or protection from predators can differ.

In species with prolonged parental care, maternal effects may extend well into the post zygotic stage. In particular, in populations where social organization exists maternal effects may be shown as cultural inheritance. In the spotted hyena (*Crocuta crocuta*) offspring 'inherit' the social rank of their mother (Engh *et al.* 1999). Red squirrels (*Tamiasciurus hudsonicus*) defend territories that contain food storage or 'cache' sites. Some females assist their offspring by incorporating additional cache sites into their territory before breeding and later bequeathing them to their offspring (Boutin *et al.* 2000).

Adaptive Maternal Effects

Maternal effects are proposed to be adaptive in a heterogeneous environment (Fox *et al.* 1997; Mousseau and Fox 1998b; Lacey 1998). The ability to manipulate offspring phenotype to suit changes in the environment may result in increased offspring survival. In this way females increase their own lifetime reproductive success.

For example, in insect species, where there is no direct parental care, pre-zygotic maternal effects are important. The choice by the female in where, how many and what size of eggs to lay is crucial to the growth and survival of her offspring. Female seed beetles (*Stator limbatus*) adjust egg size and number in response to the quality of the host plant. They lay several small eggs on the seeds of the high quality host *Acacia greggii*, and few large eggs on the poor quality host *Cercidium floridum*, seeds (Fox *et al.* 1997). Egg size is related to larvae survival on the poor quality host where larvae from large eggs have a substantially better chance of survival. On the good quality host however, almost all larvae survive to adulthood regardless of egg size.

In birds maternal effects are important not only at egg laying but also at other stages including incubation. House finches (*Carpodacus mexicanus*) have been documented as the most rapidly expanding vertebrate species of recent times (Sheldon 2002). They have colonised much of North America in the past 100 years extending their range to include areas of differing climatic conditions. Populations in differing geographic regions show adaptive differences in their sexual size dimorphism that are due to maternal effects. Variations in sex ratio with laying order and in the timing of the onset of incubation in different climatic regions have resulted in morphological differences that have enhanced offspring survival by 10-20% (Badyaev *et al.* 2002; Badyaev *et al.* 2003).

Maternal Effects and Evolution

Phenotypic variation is the raw material for natural selection. For selection to occur the phenotypic trait under selection must have a genetic basis. Maternal effects are a non-genetic means by which mothers provide their offspring with different environmental experiences that subsequently influence offspring phenotype. This suggests that maternal effects cannot be subject to selection. However, the ability of mothers to change their behaviour or level of resource provisioning suggests that the maternal effect itself must have a genetic basis within the mother. Genetic differences in mothers will result in differences in the level of maternal effects they convey which in turn will result in differing offspring phenotypes on which selection can act (Moore *et al.* 1998a). If maternal effects increase offspring survival, then the genetic basis for the effect will pass onto the offspring themselves and will be apparent in subsequent generations.

It is suggested that genetically based maternal effects can be responsible for differing evolutionary dynamics including changes to the direction of selection and in the rate of evolutionary change (Wolf *et al.* 1998; Qvarnstrom and Price 2001; McAdam and Boutin

2003). A cross fostering study on juvenile red squirrels established that growth in body mass is genetically heritable (McAdam *et al.* 2002). In a subsequent investigation it was shown that heritable maternal effects also operate resulting in changes in juvenile growth rates across one generation which were five times greater than predicted by genetic selection alone (McAdam and Boutin 2003). Similarly, maternal effects amplify the heritability of horn size and body length in dung beetles (*Onthophagus taurus*) (Kotiaho *et al.* 2003).

Differential Allocation of Maternal Effects

Life history theory describes the decisions an animal must take on how to partition resources between different traits over the course of its lifetime (Daan and Tinbergen 1997). It is predicted that due to limitations on resources, trade-offs exist between traits (Stearns 1992; Daan and Tinbergen 1997). Trade-offs are predicted to occur between current reproduction, future survival and future reproduction. Therefore, females must decide what level of resources to allocate to a particular reproductive attempt in terms of how this will affect their survival prospects and ability to invest in future reproduction.

Given that there are trade-offs in reproductive investment, females may choose to make a greater contribution towards offspring with a higher reproductive value. This investment involves maternal effects. Having an attractive mate may ultimately result in greater lifetime reproductive success if the offspring themselves are attractive. Therefore, females with an attractive mate may allocate more resources to reproduction to ensure that their offspring have the greatest possible survival chance. Burley (1988b) termed this bias in provisioning 'differential allocation'. She showed that female zebra finches (*Taeniopygia guttata*) were willing to provide more parental care, in terms of food provisioning, to nestlings fathered by an attractive male than those fathered by an unattractive one.

One crucial assumption involved in the differential allocation hypothesis is that there is a cost to the female in allocating more resources to offspring that will give a greater return on her investment. Without such a cost females might be expected to make a similar investment in all offspring regardless of circumstances. This cost is more obvious under some conditions than others and should not be neglected in studies that demonstrate differential allocation.

Differential allocation has been demonstrated in several taxa. For example, female Banggai cardinalfish (*Pterapogon kauderni*) prefer larger males and adjust both the size and number of eggs they lay in relation to male size (Kolm 2002; Kolm and Olsen 2003). Hybrid female frogs (*Rana lessonae-Rana esculenta*) reduce the number of eggs they release for

fertilization when paired to *Rana esculenta* males than when paired to *Rana lessonae* males. Reproduction with Rana *esculenta* males is less likely to be successful (Reyer *et al.* 1999).

The emphasis presented here will be on differential allocation of maternal effects in bird species.

Differential Allocation of Maternal Effects in Birds

A large number of studies have investigated resource allocation trade-offs and reproductive costs in birds. The majority of bird species are iteroparous, having more than one breeding attempt in their lifetime and their breeding cycle can easily be divided into discrete stages. Consequently, birds are useful for investigating resource trade-offs both between different reproductive attempts and between different stages of the same reproductive attempt (Reid *et al.* 2000).

All birds lay eggs that must contain the full complement of nutrients necessary for successful embryonic development and hatching. Eggs must then be incubated, and the majority of species care for their young until they reach independence. Due to the degree of parental involvement at all stages of development the potential for maternal effects, and subsequently differential allocation, in birds is large (Price 1998).

Increased investment through maternal effects in egg laying may take the form of a bias in sex ratio, laying more eggs, bigger eggs or altering egg composition. Maternal effects at incubation may involve different patterns of incubation behaviour and following hatching mothers may alter nestling provisioning.

Sex Ratio

Sex determination in birds is chromosomally based with females being the heterogametic sex. Therefore, it is theoretically possible for females to manipulate offspring sex ratio (Oddie 1998). Sex allocation theory predicts that under certain conditions the costs and benefits of producing sons and daughters could vary therefore, females should bias resources between male and female offspring in relation to which sex gives the greatest fitness return (Trivers and Willard 1973; Charnov 1982). Females mated to attractive males may bias reproduction towards sons, as the sons of attractive males may be more attractive themselves (Komdeur and Pen 2002).

Sheldon *et al.* (1999) found a positive relationship between the proportion of sons in a brood and the attractiveness of the father in blue tits (*Parus caeruleus*). Attractiveness, measured as ultraviolet reflectance on the crown, appears to act as a 'viability indicator'

predicting male survival to the following breeding season. House wrens (*Troglodytes aedon*) are polygynous therefore only good quality males are likely to breed successfully. Last hatched offspring in broods of house wrens are more likely to be female. As last hatched young in this species fledge in poorer condition it is more profitable for the parent if this particular nestling is female (Albrecht 2000).

Clutch size

Egg laying represents a sizeable investment for birds, as eggs must contain all the energy and nutrients needed by the developing embryo. The costs involved in acquiring these are far from trivial (Monaghan and Nager 1997; Monaghan *et al.* 1998). Increasing reproductive investment through laying larger clutches or bigger eggs may adversely affect survival or performance in a future-breeding attempt. Many birds use stored proteins for egg formation, most of which comes from the flight muscles (Houston *et al.* 1995). Depletion of proteins for egg formation may result in poor health, lower body condition and increased vulnerability to predators (Veasey *et al.* 2001). Female lesser black-backed gulls (*Larus fuscus*) use body reserves for egg formation. Nager *et al.* (2001) found that females induced to lay extra eggs were less likely to return to the breeding colony the following year. Those that did return were less likely to produce a clutch than control females.

Attractiveness in male peacocks (*Pavo cristatus*) is related to the degree of elaboration shown in their tail or 'train' and includes both the length of the train and the number of 'eyespots'. When mated with attractive males, peahens lay significantly more eggs (Petrie and Williams 1993). Balzer and Williams (1998) recorded a similar finding in zebra finches where females laid larger clutches for attractive males. For zebra finches in the wild, a study by Zann (1994) showed that females mated to attractive males did not lay larger clutches but laid significantly more clutches in a season than females mated to unattractive males.

Egg size

Egg size is related to chick size and subsequently chick growth and survival, particularly in the first few days after hatching when mortality is highest (Williams 1994; Christians 2002). Cunningham and Russell (2000) mated female mallards (*Anas platyrhynchos*) to attractive and unattractive males and found that females with an attractive mate laid significantly larger eggs. Female house sparrows (*Passer domesticus*) produce larger eggs when an embryo is male. House sparrows are polygynous therefore male reproductive success is related to their quality (Cordero *et al.* 2000).

Egg composition

Bird eggs are sealed environments, which at laying must contain everything the embryo needs to survive (Perrins 1996). Broadly speaking this consists of protein, lipid and water, the relative proportions of which vary depending on the life history strategy or degree of maturity of the chick at hatching (Johnson 2000). The allocation of substances of maternal origin to eggs, which may enhance the survival prospects of chicks, is currently of great interest. These substances may include antimicrobial defences, antioxidants and steroids, particularly testosterone.

Antimicrobial Defences

Egg albumen contains the antibacterial enzyme lysozyme (Burley and Vadhera 1990). However, most interest in the antimicrobial properties of eggs has centred on the yolk. On hatching the immune response of nestlings, like that of other newborn vertebrates, is relatively immature and only a relatively weak 'primary' immune response is produced (Saino *et al.* 2001; Grindstaff *et al.* 2003). Mothers can improve offspring survival and prepare them against potential infections by depositing antibodies, primarily immunoglobulins, in the yolk (Grindstaff *et al.* 2003).

Maternal antibodies may also have consequences for nestling growth as activation of an immune response may compete with nutrients and energy required for growth. Therefore, offspring with higher maternal antibody levels may be expected to show a higher growth rate, as they need not employ their own immune defences (Lochmiller and Deerenberg 2000; Grindstaff *et al.* 2003).

Immunoglobulin concentrations in eggs can vary with environmental circumstances. Gasparini *et al.* (2001) found that female kittiwakes (*Rissa tridactyla)* transferred antibodies for the spirochete *Borrelia burgdorferi* to their eggs at concentrations corresponding to local levels of infestation. Similarly, yolk antibody concentrations in the eggs of black-headed gulls (*Larus ridibundus*) correlate with nesting density, since larger bird colonies will have larger levels of intrinsic pathogens (Muller *et al.* 2004). Female barn swallows (*Hirundo rustica*) paired to attractive mates laid eggs with significantly higher levels of antibodies in first laid eggs (Saino *et al.* 2002).

Antioxidants

Antioxidants are found in both the albumen and yolk of eggs where they provide free radical protection to the egg contents and embryo during development and to the chick on hatching

(Surai 2002). Lipid soluble antioxidants are found in egg yolk and include vitamin E and carotenoids. These antioxidants can only be gained from the diet, and as they are also needed by the laying female to maintain health and body condition, they are therefore potentially limiting (Blount *et al.* 2002; Royle *et al.* 1999). Carotenoid availability has been shown to limit the egg laying capacity of lesser black-backed gulls (Blount *et al.* 2004). Since the availability of carotenoids is potentially limiting, a female may selectively allocate greater amounts to offspring with a potentially higher reproductive value to maximise her reproductive success, although this has not been investigated.

Hormones

Avian eggs contain yolk hormones of maternal origin. Yolk is the main lipid store in eggs and lipid soluble thyroid and steroid hormones including testosterone, oestrogen, progesterone and corticosterone have been identified in yolks (e.g. Schwabl 1993; Pilz *et al.* 2004). The role of yolk hormones, particularly testosterone, in embryonic and post hatching development, their effect on adult phenotype and their adaptive significance has been the subject of increasing interest in recent years.

Studies have established a relationship between the concentration of yolk hormones and embryonic and post hatching development in a small number of species. A largely beneficial effect has been demonstrated on several aspects of chick development including growth, dominance and adult morphology (e.g. Schwabl 1993; Schwabl 1996b; Eising *et al.* 2001; Pilz *et al.* 2004). Androgen levels have been found to vary within clutches in relation to laying order. Patterns of increasing and decreasing yolk testosterone levels have been found, and it has been suggested that this variation is adaptive. Increasing levels of androgens with laying order have been proposed to reduce the effects of hatching asynchrony (Schwabl 1993; Eising *et al.* 2001) whereas decreasing levels facilitate brood reduction (Schwabl *et al.* 1997c). Yolk androgen concentrations also vary between clutches (e.g. Schwabl 1997a; Reed and Vleck 2001). This behaviour is also thought to be adaptive, allowing females to alter offspring phenotype in relation to prevailing environmental conditions.

Incubation

Successful embryonic development requires a particular set of conditions within the egg during incubation including specific temperatures, humidity and a degree of physical disturbance (Reid *et al.* 2002; Gorman and Nager 2003). Parents must provide and maintain these conditions and this behaviour is costly in terms of energy expenditure, time spent sitting on the nest which

could be used for other activities such as foraging and in some species the risk of predation in attracting attention to the nest (Conway and Martin 2000).

McNamara and Houston proposed that maternal effects could be related to an animal's body condition or 'state' (McNamara and Houston 1996). Body condition may influence incubation behaviour. Birds in good condition may be able to spend more time on the nest whereas those in poor condition may spend less, as they must allocate time to foraging. Female zebra finches maintained on a supplemented prelaying diet showed longer incubation bouts during early incubation than those on a poor diet (Gorman and Nager 2003). In the wild, nest predation in zebra finches is extremely high at around 66% (Zann 1996), therefore birds on poor condition making frequent foraging trips from the nest are more likely to attract the attention of predators. Body condition is also an important predictor of incubation behaviour in the common eider (*Somateria mollissima*). Poor condition females start incubation before clutch completion. Since eider chicks hatch synchronously then chicks from later laid eggs show reduced size and growth due to a shorter incubation time, potentially reducing their survival probability (Hanssen *et al.* 2002).

Nestling Provisioning

In altricial bird species chicks hatch at an early developmental stage and require a substantial degree of parental care that includes providing food for growth and development, known as nestling provisioning. Nestling provisioning is an important determinant of chick growth and survival (e.g. Lemon 1993; Tremblay *et al.* 2003).

Two experimental studies showed that females increased nestling provisioning in relation to mate attractiveness. Female zebra finches (Burley 1988b) and female barn swallows (de Lope and Moller 1993) mated to attractive males increased the rate at which nestlings were provided with food. However, Burley's experimental design did not eliminate the possibility of assortative mating where good quality females mate with attractive males. De Lope and Moller's study is also ambiguous since attractive males reduced their nestling feeding effort. Therefore, the increase in maternal feeding may have been to compensate for the male's behaviour rather than as a result of his attractiveness.

Female blue tits alter chick feeding rates in relation to mate attractiveness (Limbourg *et al.* 2004). Females mated to males whose attractiveness had been reduced by manipulating the UV reflectance of their plumage, showed lower nestling feeding rates. Reduced nestling provisioning resulted in reduced skeletal growth of the chicks, which may be detrimental in terms of survival and competitive ability in later life. Here mate attractiveness was manipulated

after the eggs were laid thereby ruling out the possibility of assortative mating and males did not change their provisioning effort.

Aims of Thesis

Clearly, the implications for maternal effects in birds are considerable. Where possible, I have presented evidence to show that females can differentially allocate resources to offspring with a greater reproductive value. Under the differential allocation hypothesis (Burley 1988b) this increased allocation is expected to have costs to the laying female. Without this cost there would be no restriction on the female making an increased investment in all offspring regardless of the value of the reproductive attempt. Recent research has suggested that these costs may be also endured by the offspring (Muller *et al.* 2005). This thesis concentrates on the differential allocation of components of egg composition as a maternal effect, using the zebra finch (*Taeniopygia guttata*) as the study species.

Females are predicted to make a greater investment towards offspring with a higher reproductive value. Therefore, it would be expected that when paired to an attractive mate females might alter egg composition to contain a balance of components that benefits her offspring. Gil *et al.* (1999) showed that female zebra finches differentially allocated androgens to eggs laid for attractive males. The consequence of this for the developing chicks is unknown. Here, I investigated the effects of changes in egg composition due to mate attractiveness on the physiological and behavioural development of zebra finch chicks. Although I was not able to attribute the results to a particular component of egg composition I have discussed them in relation to those found in studies of yolk testosterone manipulation. Following the work of Burley (1982) attractiveness in male zebra finches was controlled using coloured leg rings. The ability of artificial ornaments to confer attractiveness on male zebra finches has been extensively investigated as described below.

Antioxidants are recognised as being vital for the successful embryonic development and post hatching survival of chicks (Surai 2002). However, the relationship between mate attractiveness and antioxidant concentration within yolk has not been investigated. I examined this relationship, again using coloured leg rings as the determinant of male attractiveness.

Finally, the mechanism by which females can alter the levels of yolk androgens in the eggs they lay is unclear. Yolk androgens may be derived exclusively from the steroidogenic activity of the follicle cells that surround the developing oocyte within the ovary. Alternatively, androgens in blood plasma may be an additional source allowing levels in yolk

to be changed. I investigated the relationship between plasma and yolk androgens across the laying cycle. Plasma androgen levels were measured using faecal sampling.

The Zebra Finch

The zebra finch (*Taeniopygia guttata*) is a small passerine found throughout most of Australia inhabiting all climatic zones across the continent from tropical to temperate areas. Their pattern of distribution is largely dependant on annual rainfall. The Australian climate is characterized by its dryness with rainfall being higher in the northern and coastal regions. Zebra finches prefer arid and semi arid habitats and are therefore found in all areas except for the northern peninsula and most of the coast.

Zebra finches are sexually dimorphic. Males and females are similar in size but vary in their plumage colouration. Female zebra finches are grey with orange beaks, broad black bands across the tail and a black 'teardrop' marking below the eyes. Males also have black tail stripes but are overall much more colourful than the females with a white abdomen, red beak, fine barring on the throat and chestnut cheek patches and flanks.

Zebra finches in the wild feed in flocks of 50 to 100 birds, preferring open grassy countryside with a scattering of trees or bushes (Zann 1996). They feed almost exclusively on dried grass seeds, rarely eating insects. Zebra finches breed in colonies of up to 40 to 50 pairs. They are socially monogamous and pair for life. Reproduction is timed to coincide with the availability of half-ripe grass seeds, which are important for the diet of both the breeding adults and nestlings (Allen and Hume 1997). They are opportunistic breeders having extended breeding seasons with the chance to raise more than one brood each season. Clutch sizes in the wild are generally 3 to 5 eggs and in captivity 4 to 6 eggs. Both parents take part in incubation and the eggs hatch after a period of about 14 days. Nestlings grow rapidly, fledging after around 17 days and become sexually mature at around 3 months old.

Zebra finches are easy to keep in captivity and breed readily when provided with the appropriate conditions. They are not only a popular cage bird but have been extensively used as an avian model species for studies in physiology and behaviour.

General Methods

Birds

The zebra finches used in these experiments were wild type and came from the stock population at St. Andrews University and from the Universities of Sheffield and Stirling.

Housing and Food

When not breeding, birds were kept in single sex cages or aviaries. Breeding birds were housed in cages measuring 228 cm x 40 cm x 40 cm and were provided with an open nestbox attached to the bars of the cage and a variety of nesting material. The lighting schedule was 16hrs light: 8hours dark with full spectrum lighting. Birds were maintained on a standard diet of mixed seed (Haith's foreign finch mix, Cleethorpes, Lincolnshire, U.K.) available *ad libitum*, supplemented twice weekly with Haith's egg biscuit and fresh spinach leaves. All birds also had access to cuttlebone, oyster shell grit and water.

Measurement of Body Condition

Condition was estimated by examining the amount of body fat. This method of measuring body condition is commonly used in passerines and involves visually scoring the amount of fat stored in the furculum between the clavicles. Scores ranged from 0 to 5, where 0 is no fat, 1 is a trace, 2 is some fat but still concave, 3 if filled but slightly concave, 4 is filled and level with the inter-clavicles, and 5 is convex and overflowing the length of the furculum (Helms and Drury 1960) (see Fig. 1).

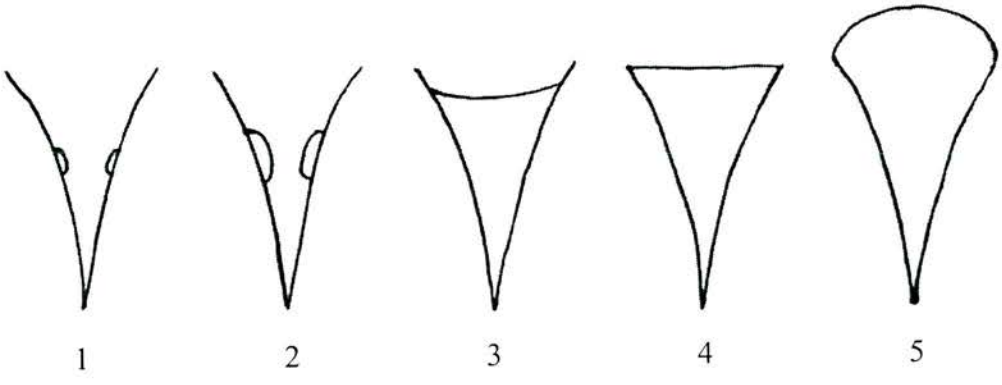


Figure1. Diagram of fat deposition in the furculum of passerine birds.

Mate attractiveness manipulation

Female zebra finches choose males on the basis of their beak colour and song rate, preferring males with redder beaks and faster song rates (Burley and Coopersmith 1987; Ratcliff and Boag 1987; Houtman 1992). These traits may indicate genetic quality (Houtman 1992; Price and Burley 1993; Price 1996). Since the purpose of these studies were to examine the influence of the mother on offspring development and behaviour it was important to eliminate the effect of male 'good genes'. Therefore it was necessary to artificially manipulate mate attractiveness. In zebra finches this is possible with the use of coloured leg rings.

The idea that leg ring colour may influence mate choice in zebra finches was first suggested in 1982 by Nancy Burley (Burley *et al.* 1982). Burley had noticed that in her experimental aviary a greater number of males wearing red leg rings were breeding compared to those wearing green leg rings. She carried out a controlled mate choice experiment examining preferences for differently coloured leg rings. The results showed that females preferred red ringed males and avoided green ringed males. Males preferred females wearing black or pink leg rings and disliked those with pale green or blue. Orange leg rings appeared neutral with both males and females not distinguishing between orange ringed and unringed birds.

Burley proposed that these preferences existed as they reflected the colours naturally found on the body of the birds. Both red and black are present, but neither males nor females show green or blue colouration on their body. The aversion to these colours may be because they suggest ill health or that the individual is from a different species. Burley went on to show that wild caught zebra finches showed the same colour ring preferences (Burley 1988a) indicating that it was not the result of domestication.

Studies using leg ring colour to manipulate male attractiveness in zebra finches have shown that ring colour influenced survival (Burley 1985), parental effort (Burley 1988b), extra-pair copulation (Houtman 1992; Burley *et al.* 1994) and dominance (Cuthill *et al.* 1997). However, other researchers have failed to find an effect of ring colour on attractiveness in zebra finches (Ratcliffe and Boag 1986; Zann 1994). There appear to be two reasons for this:
1. Many birds, including zebra finches, are capable of ultra-violet (UV) vision. Mate choice trials have shown that female zebra finches use UV in choosing a mate and prefer males seen under full spectrum lighting (Bennett *et al.* 1996; Hunt *et al.* 2001). 2. Symmetry is also important for mate choice (Swaddle 1996). Females prefer males with symmetrical plumage characteristics. This suggests that to influence mate attractiveness, the arrangement of leg rings must be symmetrical with equal numbers of the same colour on each leg (Waas and Wordsworth 1999; Bennett *et al.* 1996).

In the studies where ring colour did not affect female choice it appears that either full spectrum lighting was not used (Ratcliffe and Boag 1986) or leg rings were asymmetrical (Zann 1994). In our experiments we were careful to ensure that both these criteria were met.

Using a choice chamber we have established that the females in our zebra finch population at St. Andrews show a preference for red ringed males over green ringed ones (Two-tailed sign test n=30, x=23, below=6, equal=1, above=23, P=0.0023) (Munro, unpublished data).

2

Mate Attractiveness, Maternal Effects and Yolk Testosterone

Part One

Chick Growth and Development

The eggs of oviparous vertebrate species contain yolk hormones of maternal origin. Thyroid and steroid hormones including testosterone, oestrogen, progesterone and corticosterone are lipid soluble. Yolk is the main lipid store within eggs and these hormones have been identified in the yolks of a broad range of egg laying vertebrate species including fish (McCormick 1999), turtles (e.g. Bowden *et al.* 2001; Elf *et al.* 2002), alligators (Elf 2003), lizards (Lovern and Wade 2003) and birds (e.g. Schwabl 1993; Pilz *et al.* 2004). The role of yolk hormones in embryonic and post hatching development and their effect on adult phenotype has been the subject of increasing interest in recent years.

Avian Yolk Hormones

The majority of interest in yolk hormones has centred on avian species. The emphasis has been on how yolk hormones may be adaptive due to their influence on offspring development and phenotype. It is recognised in vertebrates that early exposure to steroid hormones during development can have permanent effects on phenotype, termed organisational effects (Arnold and Breedlove 1985; Nelson 2000). Although corticosterone (Hayward and Wingfield 2004), oestrogen (Ryan and Vanderberg 2002) and progesterone (Moore *et al.* 1998b) can cause organisational effects, most research has examined the effects of testosterone.

Steroid Hormones

Steroid hormones are formed from a common precursor – cholesterol. Through what is termed the 'hormone cascade' cholesterol is transformed, initially in the liver, to progesterone then further altered via a series of steps to androgens, corticosteroids, oestrogens and progestins depending on the secretory tissue (Becker and Breedlove 1992; Nelson 2000). Androgens, which include testosterone and its metabolites (e.g. dihydrotestosterone), are generally considered to be 'male' hormones, whereas oestrogens are often referred to as 'female' hormones. This description is misleading as both sexes produce both groups of hormones although in different proportions. In fact, androgens are the precursors of oestrogens being converted by the action of enzymes in the ovary in a process known as aromatisation. In the testis testosterone is converted to dihydrotestosterone (DHT) by the action of the enzyme 5α-reductase. As DHT is the more biologically active form of testosterone it may be important to consider the concentration of DHT together with that of testosterone in investigations of yolk testosterone concentrations.

Organizational Effects of Testosterone

In the spotted hyena (*Crocuta crocuta*) females are unusual in that they are more aggressive and socially dominant than males. In addition the female's genitalia closely resemble those of the male with a pseudopenis and pseudoscrotum. This masculinised behaviour and appearance is thought to be due to exposure to high concentrations of androstenedione during foetal development within the uterus (Glickman 1987; Nelson 2000).

Many rodent species produce litters of young resulting in several foetuses developing together in the uterus. During development an individual foetus is exposed to hormones produced by neighbouring foetuses, which diffuse through the amniotic fluid and via the maternal blood supply. The intra-uterine position (IUP) of an individual is classified according to the sex of its neighbours. For example, a foetus developing in the uterus between two male foetuses is termed a 2M foetus, whereas one developing between two female foetuses is a 0M foetus. Male foetuses produce testosterone earlier and in larger amounts than female foetuses resulting in 2M foetuses having higher concentrations of circulating testosterone than 0M foetuses. IUP results in permanent differences in physiology, morphology and behaviour in both sexes. Female house mice (*Mus musculus*) from 2M positions are more aggressive, have a shorter reproductive life span producing few male-biased litters and are less attractive to males than 0M females (Clark and Galef 1995; 1998). In gerbils (*Meriones unguiculatus*), 2M males scent mark their territory more frequently, are more attractive to females and mate more readily than 0M males (Ryan and Vanderbergh 2002).

Finally, an experimental study on the common lizard (*Lacerta vivipara*) manipulated the concentration of testosterone in the uterus resulting in the developing embryos being exposed to higher levels (Uller and Olsson 2003). Testosterone manipulated juvenile lizards grew more quickly than controls although they were also more susceptible to parasites.

Effects of Avian Yolk Hormones

Interest in the organizational effects of avian yolk hormones began in 1993 with Hubert Schwabl's study on canaries (*Serinus canaria*). Schwabl determined the concentration of testosterone in canary eggs by removing a small sample (10-15 mg) of yolk soon after laying. Testosterone concentrations were found to vary throughout the clutch, being lowest in the first laid eggs then increasing with laying order. As only a small proportion of yolk had been removed it was possible to incubate the eggs and allow them to hatch. Schwabl then examined

social rank in the canary chicks and found that it correlated with the concentration of testosterone in the yolk.

Schwabl's findings were the catalyst for a small number of experimental studies into the effects of yolk testosterone on chick development and phenotype. In these studies eggs were injected with physiological doses of testosterone bringing the overall yolk concentration to within the upper range of values naturally found in the species under study. Both behavioural and physiological effects have been recorded.

Physiological Effects of Yolk Testosterone

The time taken for an egg to hatch can indicate the rate of embryonic development. In the black-headed gull (*Larus ridibundus*) (Eising *et al.* 2001; Eising and Groothuis 2003) chicks hatched from testosterone treated eggs half a day earlier than controls. In the Chinese painted quail (*Coturnix chinensis*) (Andersson *et al.* 2004), the house sparrow (*Passer domesticus*) (Strasser and Schwabl 2004) and the canary (Schwabl 1996b) testosterone treatment did not affect hatching time. However, American kestrel (*Falco sparverius*) eggs showed delayed hatching (Sockman and Schwabl 2000).

An alternative explanation for reduced hatching time other than increased embryonic growth was suggested in a study of the *musculus complexus* or 'hatching muscle' in the red-winged blackbird (*Agelaius phoeniceus*) (Lipar and Ketterson 2000). The hatching muscle is a large dorsally located neck muscle that provides the force necessary to break the shell at hatching and also allows extension of the neck during begging. The mass of the hatching muscle in red-winged blackbird nestlings correlated with the concentration of yolk testosterone. Furthermore, experimental increase of yolk testosterone increased the mass of the hatching muscle while injection of flutamide, which prevents the action of testosterone, produced a decrease in muscle mass. In the European starling (*Sturnus vulgaris*) no relationship was established between the mass of the *musculus complexus* and the concentration of yolk testosterone (Lipar 2001).

Whereas the effects of testosterone on hatching time have been mixed, the effects on post hatching chick growth are more consistent. Chicks hatching from testosterone manipulated eggs of European starlings (*Sturnus vulgarus*) (Pilz *et al.* 2004), canaries (Schwabl 1996b) and black-headed gulls (Eising *et al.* 2001) showed increased growth over control chicks. For the American kestrel there was a negative effect of testosterone treatment with reduced nestling growth (Sockman and Schwabl 2000). This study on American kestrels is unusual in that overall the effects of testosterone treatment appear to be negative. Not only

was hatching delayed and growth reduced, but also mortality was higher. For both the European starling (Pilz *et al.* 2004) and the black-headed gull (Eising and Groothuis 2003) mortality was lower compared to controls.

Behavioural Effects of Yolk Testosterone

A limited number of studies have assessed the relationship between levels of yolk testosterone and behaviour. In developing chicks begging behaviour has been examined. Begging advertises chick need inducing parents to provide food (Kilner and Johnson 1997). Chicks that beg more often or have longer begging bouts may receive a greater quantity of food, which could in turn result in an increased growth rate. This was proposed by Schwabl (1996b) who found that canary chicks hatching from testosterone treated eggs were more likely to beg and begged more frequently within an hour of hatching than controls.

Black-headed gull chicks hatching from testosterone treated eggs also showed increased begging behaviour, being more persistent and begging more vigorously than controls (Eising and Groothuis 2003). These chicks also obtained the largest share of the food which may explain the increased growth rate of gull chicks hatching from testosterone treated eggs found in a previous study (Eising *et al.* 2001).

Additional evidence to support the idea that higher yolk testosterone levels stimulate chick begging behaviour comes from the study on the hatching muscle in red-winged blackbirds previously described (Lipar and Ketterson 2000). The *musculus complexus* not only controls hatching but also assists begging behaviour. As the mass of the hatching muscle correlates with yolk testosterone levels this could explain the increased begging behaviour found in both the canary and the black-headed gull.

Yolk Testosterone as a Maternal Effect

Although only a small number of studies have been completed on a limited number of species and the results show inconsistencies, in general higher levels of yolk testosterone appear advantageous for chick growth and development. In view of these positive effects it has been suggested that yolk testosterone may act as an adaptive maternal effect allowing the mother to influence the phenotype of her offspring, potentially increasing their fitness and her own reproductive success. In order to appreciate how this may be possible it is necessary to consider the pattern of testosterone deposition both within and between clutches.

Testosterone Patterns within Clutches

Within clutch levels of testosterone have been found to increase with laying order in several species including the red-winged blackbird (Lipar *et al.* 1999b), canary (Schwabl 1993), European starlings (Lipar 2001), common tern (*Sterna hirundo*) (French *et al.* 2001), American kestrel (Sockman and Schwabl 2000), lesser-black backed gull (*Larus fuscus*) (Verboven *et al.* 2003) and the black-headed gull (Eising *et al.* 2001). This increase with laying order has been proposed as a potentially adaptive mechanism whereby females could compensate for the effects of hatching asynchrony. Due to this, eggs hatch consecutively causing both an age and size hierarchy amongst the nestlings within a brood. As chicks are fed and grow rapidly from hatching then last hatched offspring are at a disadvantage to older, larger siblings. Depositing more testosterone in later laid eggs may be an effective strategy for females to improve offspring survival and increase reproductive success.

This hypothesis was experimentally tested in canaries (Schwabl 1996b) and black-headed gulls (Eising *et al.* 2001). Artificial clutches were created using first laid eggs to control for egg quality. The eggs were injected with different levels of testosterone to mimic those naturally found throughout the clutch with laying order. While both studies found a positive effect of testosterone on offspring growth, Eising *et al.* (2001) concluded that elevated levels of testosterone could compensate for hatching asynchrony whereas Schwabl (1996b) did not. Schwabl suggested that increased testosterone might only mitigate the disadvantages for later hatched chicks rather than fully compensate for them.

In the American coot (*Fulica americana*) (Reed and Vleck 2001), cattle egrets (*Bulbulcus ibis*) (Schwabl *et al.* 1997c) and the zebra finch (Gil *et al.* 1999) testosterone decreases with laying order. In cattle egrets it has been suggested that this pattern of testosterone allocation assists brood reduction. Cattle egrets are siblicidal. In the typical 3-egg clutch the last hatched chick is attacked and forced out of the nest or killed with bill strikes by its older siblings (Alcock 1989). The higher levels of testosterone in the first and second laid eggs are assumed to increase aggression and growth in these chicks and enhance their ability to eliminate the last hatched chick. Removal of the last hatched chick increases breeding success in cattle egrets (Mock and Ploger 1987). Broods manipulated to include 3 chicks of equal size and age fought more and fledged fewer young.

For zebra finches in the wild food supply throughout the breeding season is often unpredictable (Zann 1996). During periods of reduced food supply sibling competition, perhaps enhanced by the pattern of testosterone deposition within the clutch, may result in the death of the youngest, smallest chicks. The relationship between brood reduction and food availability is

not well documented. Zann (1996) observed that in the wild it was generally the smallest nestlings that died although no reference was made to food supply. Lemon (1993) experimentally manipulated food availability in captive zebra finches and found that brood size was reduced due to the death of the youngest chicks.

Although these explanations for the pattern of testosterone deposition within a clutch seem plausible they remain largely untested. Furthermore, testosterone levels show no pattern with laying order in tree swallows (*Tachycineta bicolour*) (Whittingham and Schwabl 2002) or in asynchronous or synchronously hatching house wrens (*Troglodytes aedon*) (Ellis *et al.* 2001).

Testosterone Patterns between Clutches

Levels of testosterone also vary between clutches. This variation can occur within species and also between clutches laid by the same female. Variation between clutches has been associated with differing environmental and social factors.

Aggression and high levels of social interaction are known to cause rises in circulating levels of testosterone in both male and female birds (e.g. Langmore *et al.* 2002). It has been suggested that increased levels of plasma testosterone in females results in higher levels of yolk testosterone in their eggs, although evidence for this is limited (Adkins-Regan *et al.* 1995; Schwabl 1996a; Hackl *et al.* 2003). Increased levels of yolk testosterone have been found to enhance dominance (Schwabl 1993; Strasser and Schwabl 2004). Therefore higher levels of yolk testosterone in relation to higher levels of aggression in the surrounding environment would produce chicks better adapted to the prevailing environmental conditions.

Several studies have found a positive correlation between nesting density, aggressive interactions and elevated levels of yolk testosterone. Two studies on house sparrows established a relationship between elevated yolk testosterone levels, the number of occupied nest sites in a colony (Schwabl 1997a) and the time spent in territory defence (Mazuc *et al.* 2003). Similarly, in the European starling (Pilz and Smith 2004), the American coot (Reed and Vleck 2001) and the tree swallow (Whittingham and Schwabl 2002) yolk testosterone levels were higher in females nesting in high-density areas where rates of aggression are greater. For black-headed gulls the opposite appears to be true as yolk testosterone levels were found to be higher in individuals nesting on the periphery of the colony where nesting density is lower (Groothuis and Schwabl 2002). However, gulls nesting on the edges of the colony tended to be more aggressive and able to hold larger territories, which may account for their increased levels of testosterone.

Laying date also significantly affects the level of clutch testosterone. In canaries (Schwabl 1996a), European starlings (Pilz *et al.* 2003) and house sparrows (*Passer domesticus*) (Schwabl 1997a) clutches laid towards the start of the breeding season had significantly higher levels of testosterone than those laid later.

Yolk Testosterone And Mate Attractiveness

An interesting situation where maternal environment can influence yolk testosterone deposition is that of mate attractiveness. Female canaries discriminate between the songs of male canaries showing preferences for different song types. Females exposed to attractive songs laid eggs with significantly higher testosterone levels than those exposed to unattractive song types (Tanvez *et al.* 2004; Gil *et al.* 2004).

Attractiveness in zebra finches can be manipulated using coloured leg rings. Males wearing red leg rings are perceived as attractive to females, whereas those wearing green leg rings are unattractive (Burley 1982; see Chapter 1 for discussion). Gil *et al.* (1999) found that females mated to red ringed, attractive males deposited significantly higher levels of testosterone in their eggs than those mated to green ringed, unattractive males.

As increased levels of yolk testosterone have been found to have a positive effect on offspring development, it is assumed that depositing more testosterone in eggs laid for red ringed males results in greater offspring fitness. This phenomenon of allocating resources in relation to mate attractiveness has been termed 'differential allocation' (Burley 1988b). Reproduction is costly since resources are limited and must be partitioned between maintaining body condition, future survival and both the present and any future reproductive attempts (Stearns 1992). Females may be willing to allocate more resources to the offspring of an attractive male assuming that those offspring will be attractive themselves thereby increasing the lifetime reproductive success of the laying female.

Aims

The purpose of this experiment was to examine the effect of elevated yolk testosterone levels due to mate attractiveness on offspring growth and behaviour in the zebra finch. Development in relation to yolk testosterone levels was assessed by comparing the progress of chicks hatching from eggs laid for red ringed and green ringed males. Within our zebra finch population we have established that males wearing red leg rings are perceived as more attractive than those wearing green leg rings (K. Munro, unpublished data).

We compared egg and clutch sizes from both experimental groups and the progress of the chicks at all stages of development including chick growth, begging behaviour, rate of maturity, final adult size, dominance, the attractiveness of male offspring and the reproductive performance of female offspring. For ease of interpretation description of the investigation has been divided into two parts. This chapter covers development for the period up to chick fledging. The following chapter describes the characteristics of the offspring as adults and ends with a summary of the findings from both chapters.

For the investigation presented in this chapter, we predicted that females laying eggs for red ringed males would lay bigger eggs and larger clutches. We expected that chicks hatching from eggs laid for red ringed males would be more likely to survive to fledging, show an increased rate of growth and greater time spent begging than chicks hatching from eggs laid for green ringed males.

Following earlier work (Gil *et al.* 1999) we assumed that females mated to red ringed males would deposit more testosterone in their eggs. Zebra finches typically lay clutches of 4 to 6 eggs. As the eggs are too small to allow yolk sampling without destroying the developing embryo, the second laid egg from each clutch was removed for hormone analysis. This would indicate the overall hormone level within the clutch. Also, using data from Gil *et al.* (1999) as a reference, the testosterone and dihydrotestosterone (DHT) concentration in the remaining eggs within each clutch could be estimated and these values used in our analysis.

Unexpectedly, we found no significant difference in the level of testosterone and DHT in second eggs laid for red ringed and green ringed males. Additionally, the estimated hormone levels had no effect on any of the physiological and behavioural traits examined and were removed from the statistical models. Our inability to show that females were depositing more testosterone in eggs laid for attractive, red ringed, males may be due to our experimental design as discussed later. As our results for levels of testosterone are ambiguous we cannot exclude the possibility that any maternal effects we have found may be due to elevated yolk testosterone. It must be pointed out that the results may also however, be due to other egg constituents that differ with mate attractiveness but in the discussion presented here we have compared our findings to those of other researchers on the effects of elevated yolk testosterone.

Methods

Female zebra finches were randomly paired with males whose attractiveness had been manipulated using red or green leg rings. Randomly allocating males and using artificially assigned attractiveness eliminates any confounding genetic effects on offspring development. Pairs were housed in individual cages as opposed to an aviary setting as this prevents the problem of assortative mating where the best quality females pair with the most attractive males.

Two experiments were carried out. The first experiment ran between March and May 2002. In order to increase the sample size and confirm the results the experiment was repeated with new birds between September and October 2002 (experiment 2). In both experiments 32 pairs were set up initially. Several pairs laid clutches and subsequently destroyed them. These clutches were included in the egg weight analysis and the pairs were replaced with new birds taken from the stock population.

Clutches were cross fostered between pairs during early incubation matched as closely as possible in terms of laying date and clutch size. Cross fostering involved exchanging eggs between the nests of males with the opposite ring colour as well as males with the same ring colour. Table 1 shows the number of clutches cross fostered between nests according to male ring colour and the total number of clutches that successfully hatched at least one chick from each of the groups.

Male Status	No. Of Clutches Cross-fostered		No. Of Surviving Clutches	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
Green ringed to Green ringed	6	4	4	2
Green ringed to Red ringed	7	10	5	7
Red ringed to green ringed	6	15	3	11
Red ringed to Red ringed	6	2	4	2
Total	25	31	16	22

Table 1. Cross fostering of clutches in experiments 1 and 2.

Cross fostering allows the separation of pre natal (egg composition) maternal effects from post natal (nestling provisioning) maternal effects. An alternative experimental design which would have allowed post natal maternal effects to have been eliminated is cross fostering to a control group of males wearing neutral orange leg rings. However, this design was not feasible due to the large number of birds which would have been involved. A distinction was made between pre natal and post natal maternal effects during the statistical analysis by using male ring colour and foster male colour as variables within the models.

Housing and dietary conditions are described in chapter 1. Where possible females with no previous breeding experience were used thereby eliminating any potential carry over effects of male attractiveness from earlier breeding attempts (Rutstein *et al.* 2004b; Rutstein *et al.* 2005). The remaining females had bred with males whose attractiveness had not been manipulated.

Egg Size

Eggs were weighed and measured on the day of laying, marked with a non-toxic pen and returned to the nest. Second laid eggs were removed, replaced with a dummy egg, and frozen at –20°C awaiting hormone analysis.

Yolk Androgen Extraction and Assay

Extraction

To extract testosterone (T) and dihydrotestosterone (DHT) yolk samples were weighed and mixed with 0.5ml distilled water. Androgens were extracted twice by homogenizing with 3ml diethyl ether. The ether supernatant was decanted following snap freezing in a dry-ice acetone bath then dried using a vacuum centrifuge. The residue was re-dissolved in 2ml 90% ethanol and left overnight at –20°C. Precipitated proteins and lipids were separated from the ethanol phase by decanting following centrifuging at high speed for 6 minutes. The ethanol was dried in a vacuum centrifuge and 2ml assay buffer was added to the residue. Of this 1.5ml was removed for the total androgen (T and DHT) assay. The remaining 1ml underwent an oxidation step. This oxidises T leaving DHT only remaining allowing a DHT assay to be carried out on the final extract. The oxidation step involved adding 100µl oxidising reagent and 0.5ml distilled water and incubating at room temperature for 20 minutes. The samples were then extracted twice with 3ml diethyl ether as before, dried and dissolved in 1ml assay buffer.

Assay

Radioimmunoassays were carried out to determine the total (T and DHT) androgen content and the DHT content of yolk samples. The methodology followed that in the commercially available RIA kit from Amersham (Amersham-Pharmacia Biotech, Buckinghamshire, U.K.), using 200µl tritiated DHT (5µl in 80ml assay buffer), 200µl extracted yolk sample, and 200µl anti-T rabbit antiserum at 1/6000 dilution (T-DHT cross reactivity of 45-50%). Standard curves for total (T and DHT) and DHT assays were produced using T (12.5-400pg/ml) and DHT (25-800pg/ml) standards respectively.

Extraction recovery efficiencies were calculated using pooled yolk samples containing a known quantity of radiolabelled T or DHT. Extraction efficiency for total assay was 80% and for DHT it was 60%. Parallelism shows that there is a linear relationship between the concentration of the target hormone in the samples and the assay values obtained. This was confirmed by running serial dilutions of yolk extracts demonstrating that samples dilute parallel to the standard curve. Accuracy of the assay is defined by intra-assay and inter-assay coefficient of variation (CV). The intra assay CV was found to be 4.3 $\pm$ 0.3% (mean $\pm$ s.e.) for T and 6.3 $\pm$ 0.6% for DHT. The inter assay CV was 17 $\pm$ 2.2% for both.

Begging

In experiment 1 begging was examined at three, four and five days after hatching. In experiment 2 we recorded begging behaviour on days two, three and four. These stages were considered appropriate, as the chicks are strong enough to be removed from the nest and beg well but have not yet opened their eyes.

Chicks were removed from the nest individually and placed on a heated pad. They were fed to satiation with Haith's egg biscuit and left on the heating pad for one hour. This allowed all chicks to be at the same hunger level when tested. After the hour, begging behaviour was measured for each chick individually over a three-minute trial. Chicks were tapped once on the beak with a plastic pipette tip (size for 1ml Gilson pipette) to elicit a begging response. When begging had stopped, or if a chick failed to beg, a count of three seconds was made before its beak was tapped again. Each session was recorded on 8mm tape using a Sony handycam video camera. The chick was then fed to satiation and returned to the nest.

Analysis of begging behaviour was determined from the videotapes by measuring the total time each chick spent begging within the 3 minute trial using a stopwatch. Begging was defined as when the chick held its mouth open with no distinction made of begging posture.

Growth

In experiment 1, chicks were weighed on an electronic balance (± 0.01g) on hatching, then daily until they fledged at around 18 days old. Tarsus measurements were taken daily using digital callipers (±0.01mm) from day four until fledging. Tarsus measurements before day four were found to be unrepeatable. Growth followed a sigmoid curve with the period of most rapid growth, and the linear part of the curve, falling between days 4 to 14. For analysis growth was defined as the change in tarsus length in millimetres or weight in grams between days 4 and 14. In experiment 2 chicks were weighed on hatching, then weight and tarsus measurements taken and at days 4, 14 and 19.

Survival

Survival was measured as those offspring that had fledged. Non-survivors were chicks who had hatched but died before reaching fledging.

Statistical Analysis

General linear models were used for statistical analysis since they can be used to examine the effects of multiple explanatory variables simultaneously where those variables are both continuous and categorical. Simple general linear models were used in the analysis of clutch size, DHT and testosterone concentrations. For egg size, time spent begging and growth, mixed general linear models were used since it was necessary to include a random factor. This takes into account that several eggs or chicks may originate from a single female and are subsequently related. They are therefore 'nested' within that female and are not treated as independent measures. Non-independence within the data contravenes the assumptions of statistical tests (Grafen and Hails 2002). In all cases nest or female of origin was used as the random factor. Analysis of survival used a generalized mixed linear model, since here the data was binomial.

Analysis followed a backwards-stepwise procedure. All biologically relevant variables and interactions were included in the initial model. Variables and interactions were subsequently removed if their probability was greater than P=0.1. Final models were checked for 'goodness of fit' using residual plots (Grafen and Hails 2002). Normality of error was

tested through examination of normal probability plots and histograms of the residuals. Heterogeneity of variance was examined by plotting the residual versus the fitted values. Statistical analysis used SAS version 8e. Data were tested for normality using Kolmogorov-Smirnov tests on Minitab version 12 and transformed where necessary.

It became apparent during the analysis that significant differences existed between the two experiments. For this reason the experiments were analysed separately and then together with experiment number as an explanatory variable.

Explanatory Variables

Male ring colour

Male ring colour was used in the analysis of egg size and clutch size. Fathers ring colour was included in the analysis of chick traits to identify maternal effects due to egg composition and foster fathers ring colour determined maternal effects due to nestling provisioning.

Egg Mass

Egg mass in grams was used in the analysis of egg size. Egg volume, calculated from Hoyt's formula (Hoyt 1979), was not used. Mass is likely to be a more accurate indicator of size than volume as Hoyt's formula assumes all eggs to be of a standard shape. Also, mass and volume could not both be used since they are not independent measures: regression of egg mass and volume for both experiments together show an r-squared value of 0.62.

Laying Order and Hatching Order

Eggs were usually laid one each day until the clutch was complete and generally hatched one each day in the order of laying. As hatching and laying order are very similar they were not used simultaneously in the analysis. A regression of hatching order and laying order showed r-squared values of 0.57 for experiment 1, and 0.73 for experiment 2. Therefore, models were run using both laying order or hatching order in separate models and the results for the variable with the lowest probability score is presented.

Chick Sex

Chick sex was determined at maturity. The experiments produced eight white morph chicks. Male and female white morph zebra finches differ only in their beak colour therefore the sex of these chicks was determined using molecular techniques described in chapter 3.

Female Mass

Female mass measured in grams represented the size and body condition of the laying female. Tarsus was not used, as measurements were not taken for experiment 1. Measurements of body fat (see chapter 1) were taken for both experiments but were not used as they are related to mass: experiment 1 r-squared value 0.41, experiment 2 r-squared value 0.23.

Broodsize

Broodsize defined the number of chicks remaining within each nest or brood from day 4 onwards, i.e. covering the period of rapid growth onwards. Most mortality occurred before this time.

Additional variables specific to the analysis of individual traits are described as appropriate.

Results

In experiment 1, 33 females consisting of 16 mated to green ringed males and 17 mated to red ringed males laid eggs. In experiment 2, 32 females including 18 mated to red ringed males and 14 mated to green ringed males bred successfully. In both experiments data from the two heaviest females were removed from the analysis. These females were outliers as they weighed well over 20g and a regression of mass against fat score showed standardized residuals which were both large and influential (Experiment 1: female 1, 21.2g, standardized residual = 2.26; female 2, 25.85g, standardized residual = 2.84. Experiment 2: female 1, 22.01g, standardized residual = 2.54; female 2, 23.38g, standardized residual = 2.21).

Due to the complexity of the analysis and the number of variables used the results presented here have been simplified to present key findings only. Full tables of results for all variables within each model are given in the results appendix at the end of the chapter.

Egg Size

Experiment 1

Variables used were laying order, female mass and male ring colour. Sample size 160 eggs.

There was a significant interaction between laying order and male ring colour ($F_{1, 128}$= 4.71, P=0.0318). Egg mass increased with laying order for both groups but the increase was greater for females mated to red ringed males (Fig. 1a).

Figure 1a. Fitted mean egg mass (g ± s.e.) with laying order and male ring colour for experiment 1.

There was a non-significant trend for heavier females to lay larger eggs ($F_{1, 128}$, P=0.0569) (Fig.1b).

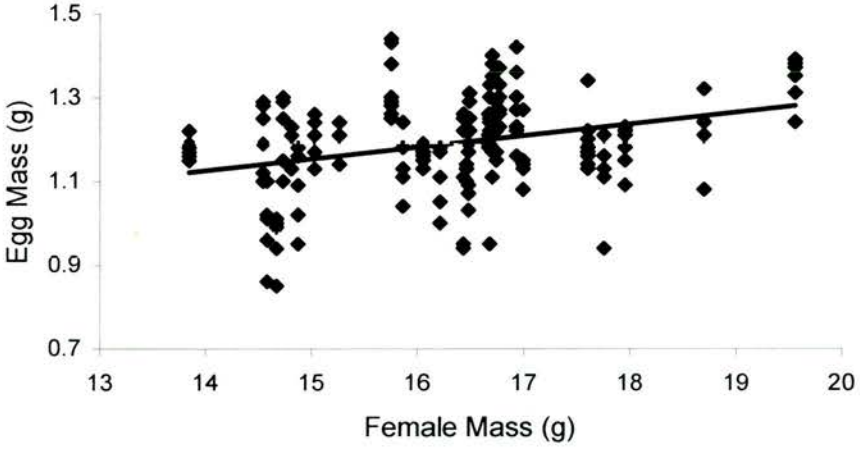


Figure 1b. Egg mass (g) and female mass (g) for experiment 1.

Experiment 2

Variables used were laying order, female mass and male ring colour. Sample size 147 eggs.

Male ring colour had no effect on egg mass in experiment 2 ($F_{1, 28}$=2.21, P=0.1486). There was a highly significant increase in egg mass with laying order ($F_{1, 116}$= 10.28, P=0.00017). (Fig.1c). Female mass had no effect on egg mass ($F_{1, 116}$ =0.02, P=0.8793).

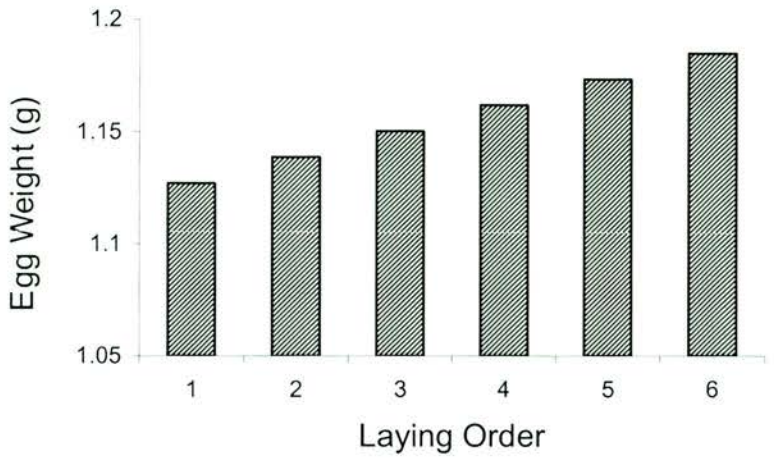


Figure 1c. Fitted mean egg mass (g) with laying order for experiment 2.

Experiment 1 and 2 Together

Variables used were laying order, female mass, experiment number and male ring colour. Sample size 307 eggs.

Analysis of both experiments showed differences between them. An interaction between experiment number and laying order ($F_{1, 253}$= 7.36, P=0.0071) (Fig. 1d) indicated that egg mass increased with laying order in both experiments, but females laid heavier eggs in experiment 1.

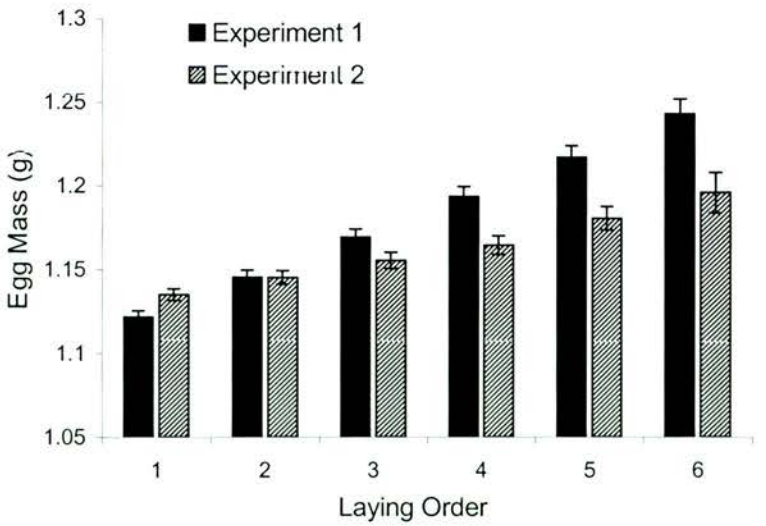


Figure 1d. Fitted mean egg mass (g ± s.e.) with laying order for experiments 1 and 2.

Female mass had a significant effect on egg mass for experiments 1 and 2 together ($F_{1, 253}$ =4.42, P=0.0365). Heavier females laid larger eggs (Fig. 1e).

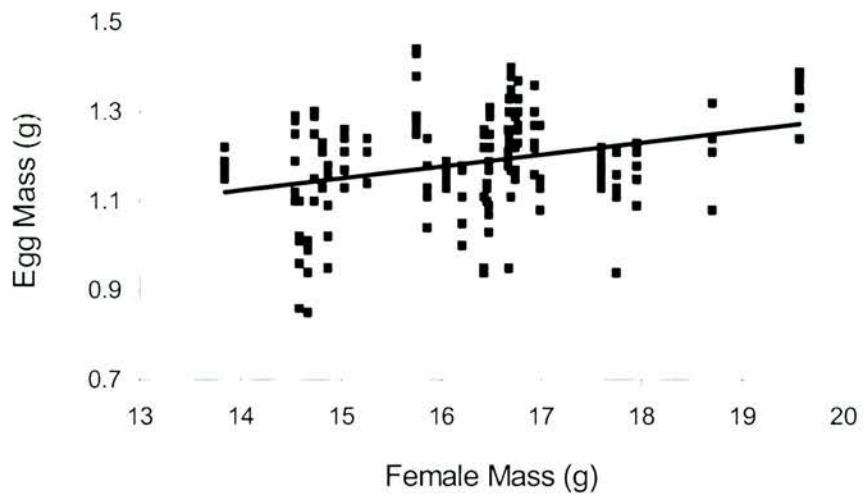


Figure 1e. Egg mass (g) and female mass (g) for experiments 1and 2.

Clutch Size

Variables used were male ring colour and female mass. Sample size was 29 clutches for experiment 1 and 30 clutches for experiment 2.

There was no effect of any of the variables examined on clutch size in experiment 1 or experiment 2 when analysed separately.

Experiments 1 and 2 Together

Variables used were male ring colour, experiment number and female mass. Sample size was 62 clutches.

Clutch size differed significantly between the two experiments ($F_{1, 10.63}$ = 9.37, P=0.0033). Females laid larger clutches in experiment 1 (Fig. 2a).

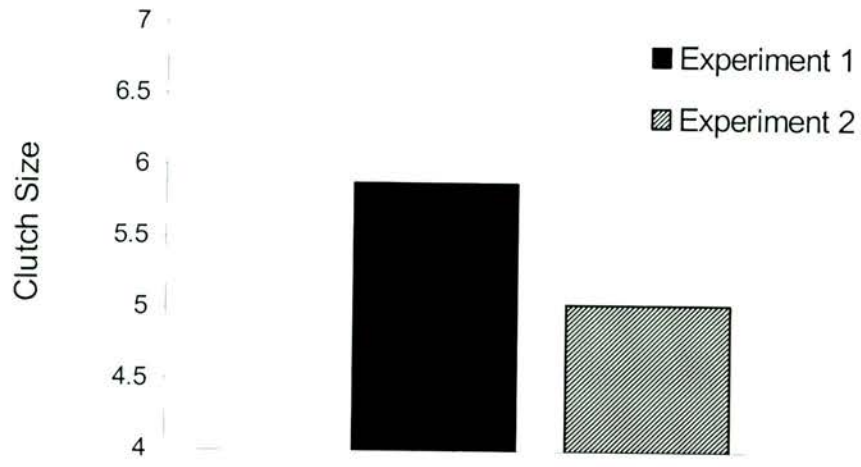


Figure 2a. Fitted mean clutch size for experiments 1 and 2.

Female mass showed a non-significant trend ($F_{1, 4}$ =4.42, P=0.0531) with heavier females laying larger clutches (Fig. 2b).

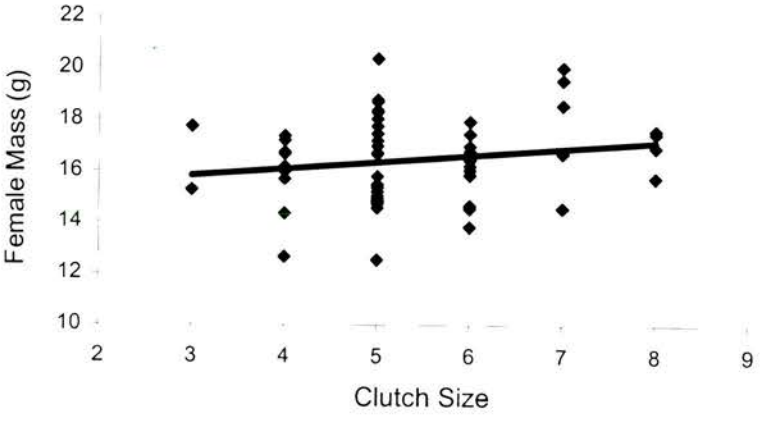


Figure 2b. Fitted mean female mass (g) and clutch size for experiments 1 and 2.

Yolk Hormones

Variables used were male ring colour, clutch size and female mass. Sample size was 30 eggs for experiment 1 and 24 eggs for experiment 2.

There was no effect of male ring colour, clutch size or female weight on either DHT or testosterone concentration in yolk in either experiment 1 or 2 when analysed separately. It was not possible to analyse experiments 1 and 2 together as the assays to determine the hormone concentrations in each experiment were run independently and were therefore not comparable.

Time Spent Begging

Experiment 1

Variables used were fathers ring colour, foster fathers ring colour, chick sex, hatching order, laying order, broodsize and female mass. Sample size was 43 chicks.

Chicks with red ringed fathers begged for longer (Fig. 3a) ($F_{1, 15}$=7.56, P=0.0149). Neither foster father's ring colour ($F_{1, 13}$ =0.49, P=0.4979) nor broodsize ($F_{1, 25}$=0.01, P=0.9061) showed any effect on time spent begging.

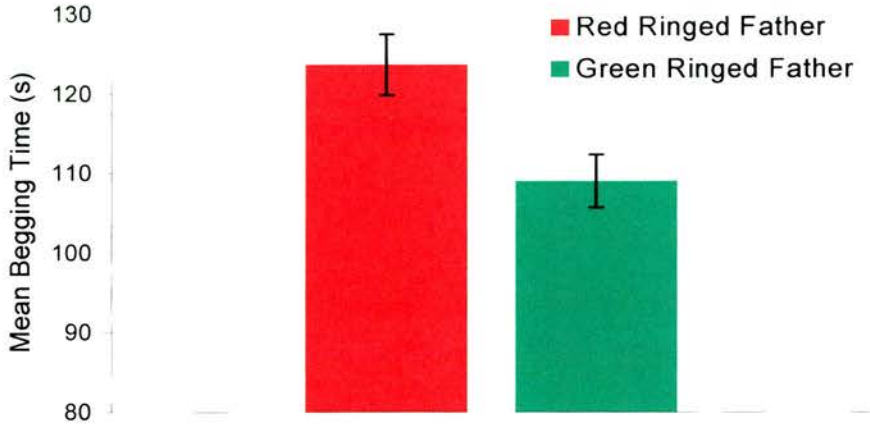


Figure 3a. Fitted mean time spent begging (s $\pm$ s.e.) with fathers ring colour for experiment 1.

Experiment 2

Variables used were fathers ring colour, foster fathers ring colour, chick sex, hatching order, laying order, broodsize and female mass. Sample size was 48 chicks.

Time spent begging was significantly affected by both father's ring colour ($F_{1, 17}$ = 12.07, P=0.0029)(Fig. 3b) and foster father's ring colour ($F_{1, 17}$ =7.64, P=0.0133)(Fig. 3c). Chicks with green ringed fathers and green ringed foster fathers begged for longer.

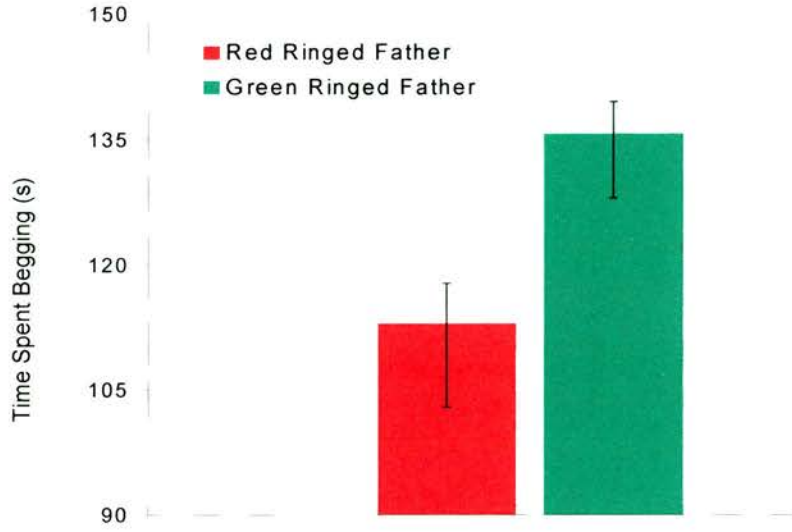


Figure 3b. Fitted mean time spent begging (s ± s.e.) with fathers ring colour for experiment 2.

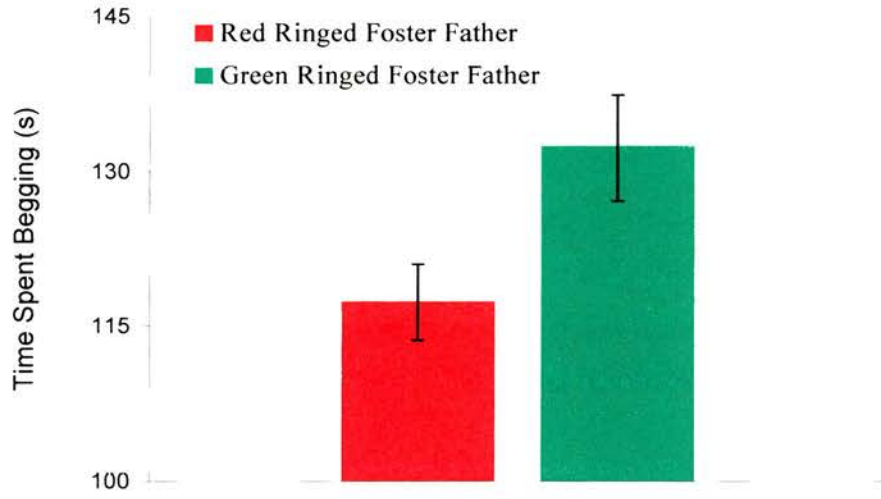


Figure 3c. Mean time spent begging (s ± s.e.) with foster fathers ring colour for experiment 2.

Chicks in larger broods in experiment 2 spending less time begging ($F_{1, 34}$ =6.57, P=0.0150) (Fig. 3d).

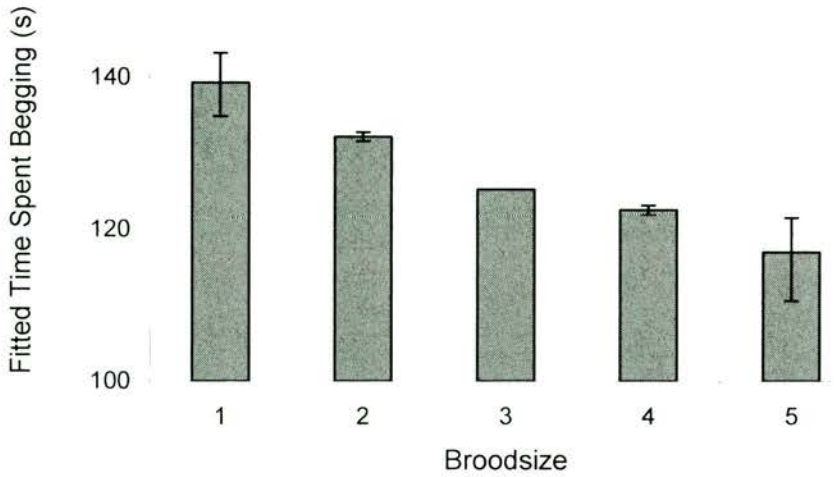


Figure 3d. Fitted mean time spent begging (s ± s.e.) with brood size for experiment 2.

Experiments 1 and 2 Together

Variables used were fathers ring colour, foster fathers ring colour, chick sex, hatching order, laying order, broodsize, experiment number and female mass. Sample size was 100 chicks.

An interaction between father's ring colour and experiment number ($F_{1, 64} = 30.29$, P=<0.0001) indicated there was a highly significant difference in mean time spent begging between experiments in relation to father's ring colour (Fig. 3e). In experiment 1 chicks with red ringed fathers begged for longer whereas in experiment 2 chicks with green ringed fathers begged for longer.

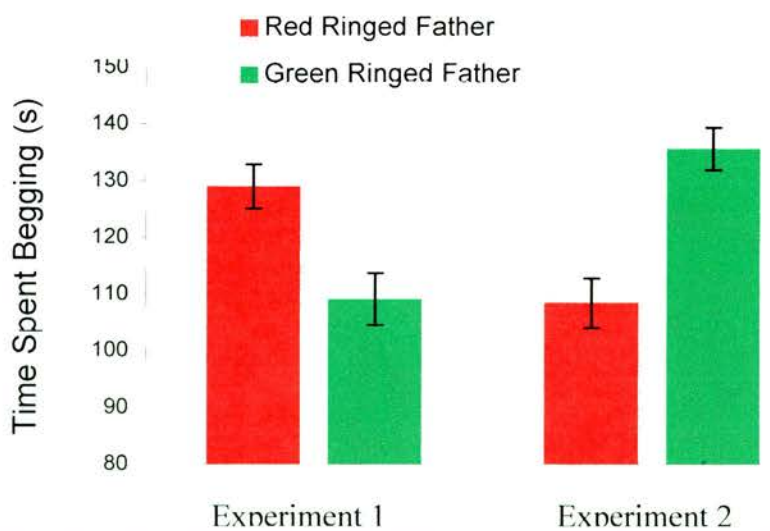


Figure 3e. Mean time spent begging (s ± s.e.) with fathers ring colour for experiment 1 and 2.

There was also a significant interaction between foster father ring colour and experiment number ($F_{1, 64}$=5.56, P=0.0214). Chicks with green ringed foster fathers begged for longer in experiment 2, whereas in experiment 1 there was no difference in the time spent begging with foster father's ring colour (Fig. 3f).

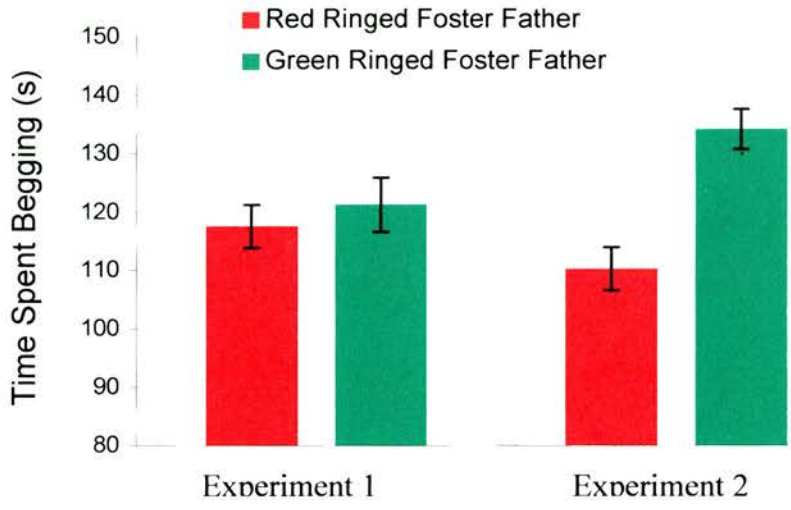


Figure 3f. Mean time spent begging (s ± s.e.) with foster fathers ring colour for experiments 1 and 2.

Growth

Weight Gain

Variables used were fathers ring colour, foster fathers ring colour, laying order, hatching order, female mass, chick sex and broodsize. Sample size was 42 chicks for experiment 1 and 42 chicks for experiment 2.

There was no effect of any of the variables examined on weight gain in experiment 1 or 2 when analysed separately.

Experiment 1 and 2 Together

Variables used were fathers ring colour, foster fathers ring colour, laying order, hatching order, female mass, chick sex, experiment number and broodsize. Sample size was 91 chicks.

Chicks in experiment 2 gained significantly more weight than chicks in experiment 1 ($F_{1, 60}$=13.8, P=0.0004) (Fig 4a.). Hatching order also had a significant effect on weight gain ($F_{1, 60}$=4.71, P=0.0340) (Fig. 4b) showing a decrease with hatching order.

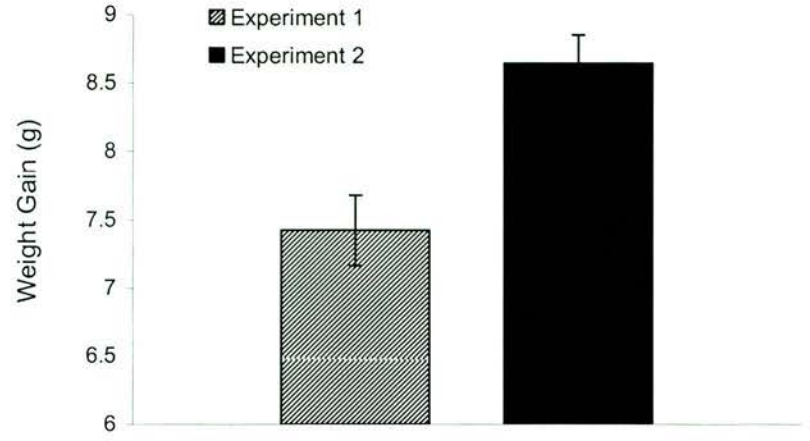


Figure 4a. Fitted mean weight increase (g ± s.e.) for experiments 1 and 2.

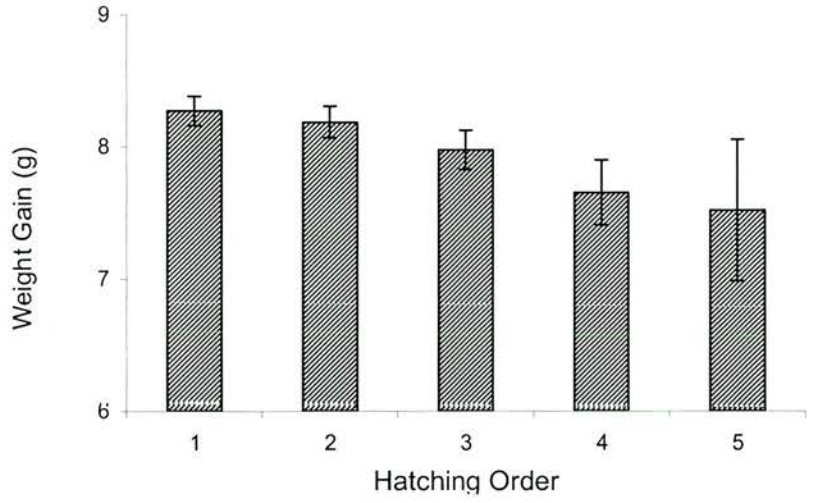


Figure 4b. Fitted mean weight gain (g ± s.e.) with hatching order for experiments 1 and 2.

Foster father's ring colour showed a non-significant trend. Chicks with red ringed foster fathers tended gained more weight ($F_{1, 32}$ =3.66, P=0.0647) (Fig. 4c.).

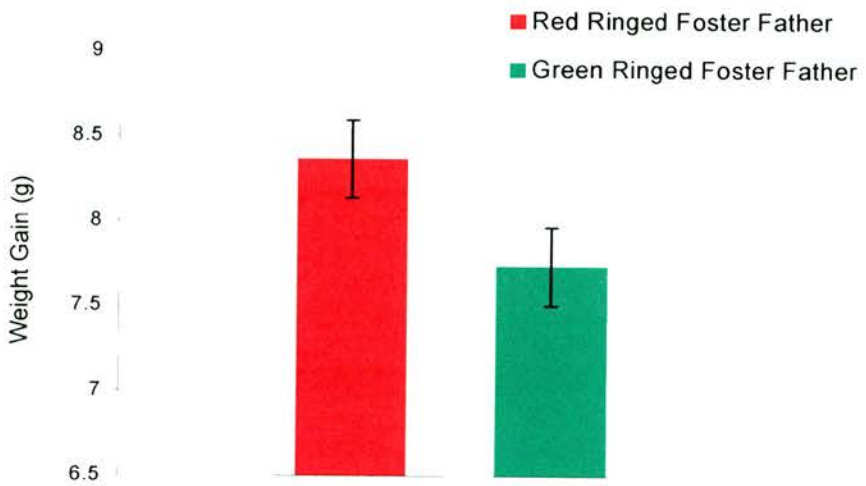


Figure 4c. Weight gain (g ± s.e.) with foster fathers ring colour for experiments 1 and 2.

Tarsus Increase

Experiment 1

Variables used were fathers ring colour, foster fathers ring colour, laying order, hatching order, female mass, chick sex and broodsize. Sample size was 44 chicks.

An interaction between father's ring colour and laying order ($F_{1, 26}$ =9.39, P=0.0050) showed that for chicks with green ringed fathers tarsus increase declined with laying order (Fig 5a) but was relatively unchanged for chicks with red ringed fathers with laying order.

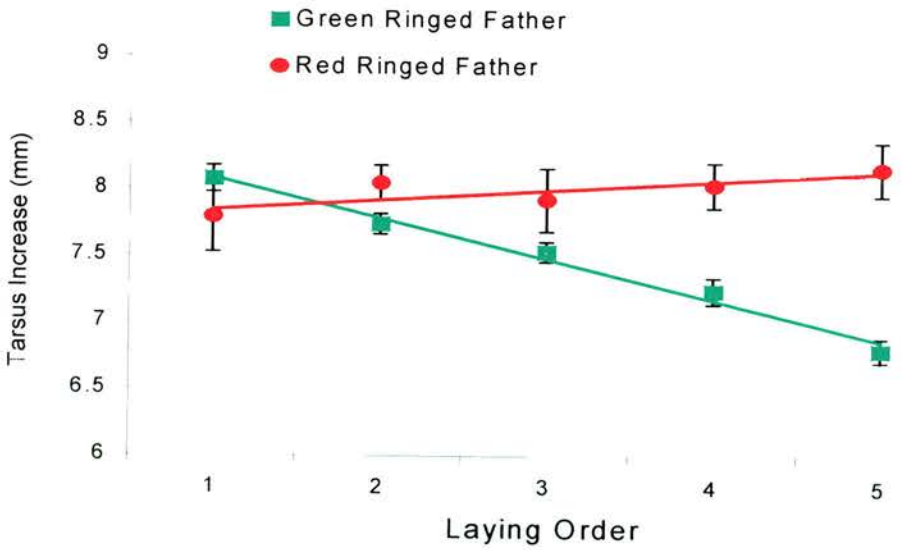


Figure 5a. Fitted mean tarsus increase (mm ± s.e.) with father's ring colour and laying order for experiment 1.

Brood size also had an effect on tarsus increase. Chicks from larger broods had a greater tarsus increase ($F_{1, 26}$ =4.73, P=0.0389) (fig 5b.).

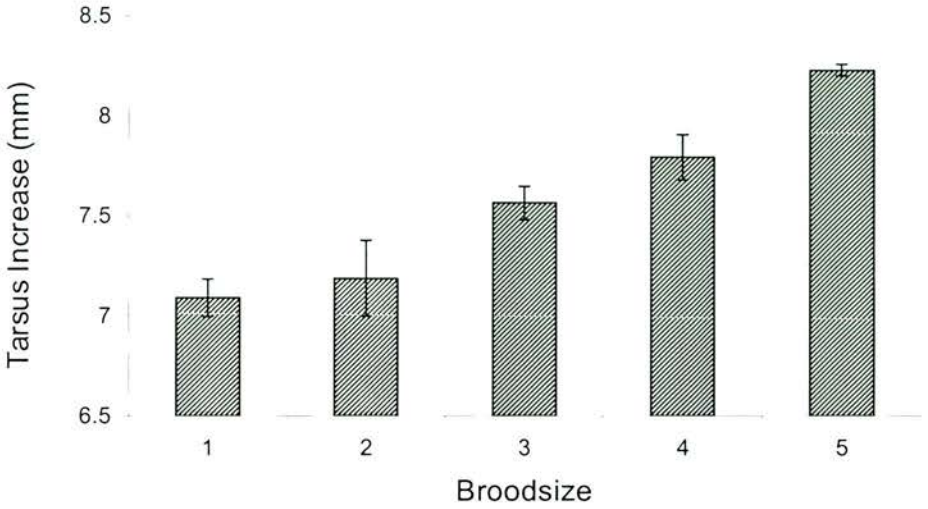


Figure 5b. Fitted mean tarsus increase (mm ± s.e.) with brood size for chicks in experiment 1.

Experiment 2

Variables used were fathers ring colour, foster fathers ring colour, laying order, hatching order, female mass, chick sex and broodsize. Sample size was 44 chicks.

In experiment 2, tarsus increase showed no relationship with father's ring colour ($F_{1, 16}$ =0.25, P=0.6229) or brood size ($F_{1, 28}$= 0.07, P=0.7984). Hatching order had a significant effect with later hatched chicks growing more slowly ($F_{1, 28}$ =8.14, P=0.0080) (Fig. 5c).

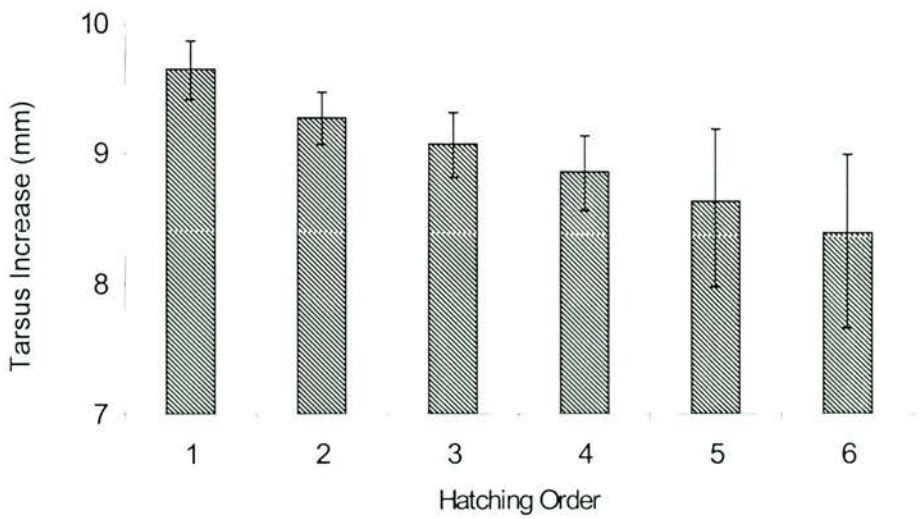


Figure 5c. Fitted mean tarsus increase (mm ± s.e.) with hatching order for experiment 2.

Experiments 1 and 2 Together

Variables used were fathers ring colour, foster fathers ring colour, laying order, hatching order, female mass, chick sex, experiment number and broodsize. Sample size was 91 chicks.

Chicks with green ringed fathers showed a decline in tarsus growth with hatching order whereas chicks with red ringed fathers had similar growth across hatching order ($F_{1, 57}$ =8.92, P=0.0042) (Fig. 5d.).

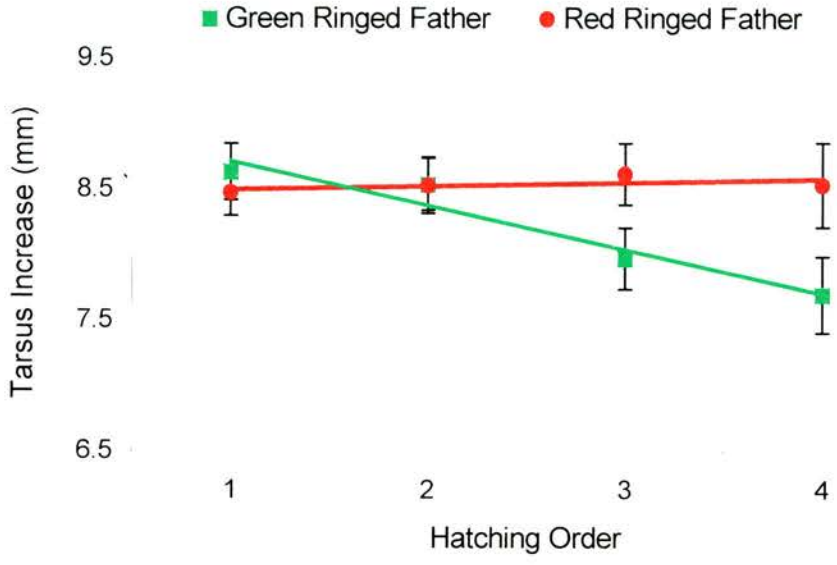


Figure 5d. Fitted mean tarsus increase (mm ± s.e.) with hatching order for experiments 1 and 2.

Tarsus increase was significantly different between experiment 1 and 2 ($F_{1, 57}$ =35.02, P=<0.0001) (Fig. 5e). Tarsus growth was faster in experiment 2.

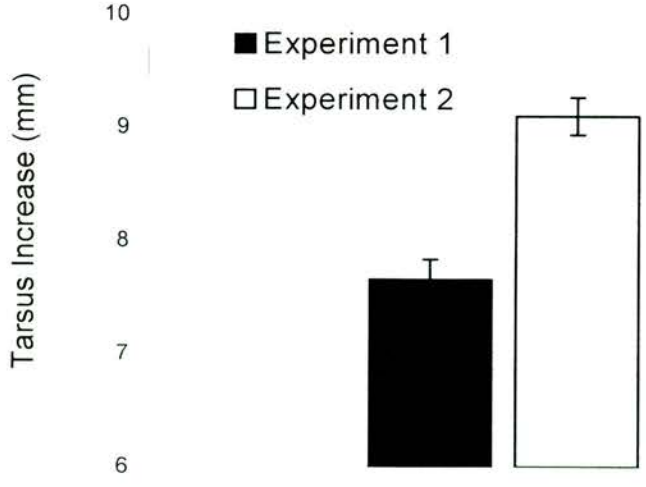


Figure 5e. Fitted mean tarsus growth (mm ± s.e.) for experiments 1 and 2.

Survival

Variables used were fathers ring colour, foster fathers ring colour, female mass, broodsize, laying order, hatching order and hatching weight. Hatching weight was included in this analysis as it relates to egg weight which has been shown to be an important indicator of survival in the first few days after hatching. Sample size was 62 chicks for experiment 1 and 66 chicks for experiment 2.

Male ring colour had no effect on survival in experiment 1 ($F_{1, 20}$ =0.73, P=0.4014), experiment 2 ($F_{1, 21}$ =0.61, P=0.4424) or when both experiments were analysed together ($F_{1, 43}$ =2.18, P=0.1473).

Discussion

A large number of results are presented here. In order to keep the discussion manageable it will be divided into three sections presenting the main groups of findings.

1. Differences between Experiments

There are significant differences between the results of experiment 1 and experiment 2. The experiments were carried out indoors under identical conditions of artificial daylength and temperature. The only difference between the experiments was the season in which they were run. Opportunistic species have been traditionally considered non seasonal breeders, taking advantage of favourable conditions as they arise, however they do appear to show an underlying seasonality (Hahn 1998; Hau *et al.* 2004). Although zebra finches are opportunistic breeders they are influenced by seasonality (Zann 1994; Bentley *et al.* 2000; Zann and Runciman 2003). Researchers have suggested that endogenous circannual rhythms may contribute to seasonality in tropical and opportunistic species (Gwinner and Dittami 1990; Gwinner 1996; Dawson 2003; Moore 2005). Endogenous circannual rhythms are innate biological calendars which control annual cycles of behavioural and physiological functions such as reproduction, moult and migration (Gwinner and Dittami 1990; Gwinner 1996).

Clutch Size

Clutch size was significantly greater in experiment 1 (Fig. 2a) which was completed in the springtime (March to May 2002). Experiment 2 was completed in the autumn (October to December 2002). A seasonal change in clutch size is well documented in both single and multiple brooded bird species (Rowe *et al.* 1994; Gil-Delgado *et al.* 2005). Zebra finches are a

multiple brooded species with an unusually long breeding season in the wild of up to 10 months (Zann 1996). The peak of breeding is in the springtime with clutches significantly smaller towards the end of the breeding season in the autumn (Zann 1996).

Egg Size

In both experiments egg mass increased with laying order. This increase was greater in experiment 1 resulting in later laid eggs in experiment 1 being heavier than those in experiment 2 (Fig. 1d).

Although it appears that females in experiment 2 are laying smaller eggs, it is possible that they are simply not making any additional investment in egg size with male ring colour. Further statistical analysis supports this. All data on egg mass with laying order from experiment 2 were compared with data from experiment 1 of eggs laid for either red ringed or green ringed males only. There was no difference between the mass of eggs in experiment 2 with eggs laid for green ringed males in experiment 1 ($F_{1, 180}$ =1.14, P=0.2874). However, there was a significant difference between the mass of eggs in experiment 2 with those laid for red ringed males in experiment 1 ($F_{1, 193}$ =16.84, P=<0.0001). Eggs laid for red ringed males in experiment 1 were larger than eggs laid for all males in experiment 2. This suggests that females in experiment 2 did not lay smaller eggs, but that unlike the females in experiment 1, they did not make an extra investment by laying larger eggs when mated to a red ringed male.

Begging

Both Schwabl (1996b) and Eising and Groothuis (2003) found that increased levels of yolk testosterone enhanced begging behaviour. In support of this, in experiment 1 chicks with red ringed fathers begged for longer (Fig. 3e). However, in experiment 2 we found the opposite result where chicks with green ringed fathers begged for longer. The results for foster father's ring colour further confuse the issue. In experiment 1 there is no difference in the time spent begging by chicks with red ringed and green ringed foster fathers, whereas in experiment 2 chicks with green ringed foster fathers begged for longer (Fig. 3f).

There is no straightforward explanation for these conflicting results although several points can be made. The statistical analysis from experiment 1 where chicks with attractive fathers begged for longer did not show normal residuals suggesting that it does not provide a good explanation of the data and may therefore be unreliable (Grafen and Hails 2002). An additional unexplored variable may provide a better fit or it may be that the relationship

between father's ring colour and time spent begging is not linear. Lengthy manipulation of both the variables and data did not improve this result.

The validity of our results on begging behaviour may be questionable as it is unclear from the literature what differences in begging behaviour actually indicate. Begging behaviour in altricial bird species undoubtedly signals need, inducing the parents to provide food. However, there is considerable debate as to whether begging is an honest indicator of need or whether it can be a manipulative behaviour (Kilner and Johnstone 1997; Royle *et al.* 2002). Learning may also shape begging behaviour (Kedar *et al.* 2000; Rodriguez-Girones *et al.* 2002). Nestlings can learn to adopt the begging strategy that gains the most rewards and this may depend on sibling competition and parental feeding strategies. In an experiment on house sparrows Kedar *et al.* (2000) showed that chicks could readily modify their begging levels within a few hours in response to altered parental feeding strategies. Parent birds differ in their feeding strategies; some provision all chicks within the brood equally (Kilner and Johnstone 1997) whereas others preferentially feed the largest chicks. Begging behaviour therefore appears to be complex and difficult to interpret.

Our experimental procedure for measuring begging behaviour may not have been the most appropriate method. We measured begging behaviour in a laboratory setting using chicks of between 2 and 5 days old. Schwabl (1996b) measured chick begging within an hour of hatching. The intensity of begging behaviour at hatching may be a better measure for two reasons. Firstly there is no opportunity for the chick to have learned to adopt a particular begging strategy. Secondly, begging behaviour at hatching may be an important measure of the probability of chick survival as this is a critical time when mortality is high. Chicks must ensure that parent birds begin to provide food. In our experiments it was apparent that most chick mortality was due to parents not initiating feeding.

As we used an artificial setting for our experiment we were also not able to get an indication of how much food each chick was likely to gain as a result of its begging strategy. Eising and Groothuis (2003) measured begging behaviour in the field where they were able to observe interactions between nestmates and record the amount of food obtained by each chick.

Growth

Chicks in experiment 2 grew more quickly in terms of both weight gain and tarsus increase than chicks in experiment 1 (Fig. 4a and Fig.5e). The reasons for these differences are unclear. Differences in both the quantity and quality, in terms of protein content, of nestling

diet in zebra finches have been shown to affect the rate of nestling growth (Boag 1987; Skagen 1988; Haywood and Perrins 1992; Birkhead *et al.* 1999). It is unlikely that alterations in food quality or quantity caused the differences in growth rate between experiments. Food was available to all breeding birds *ad libitum* with identical levels of food quality between experiments. Furthermore, in a study of chick development carried out using two identical experimental replicates Gorman and Nager (2004) also found unexplained differences in chick tarsus increase and weight gain.

2. Effects of Male Ring Colour

Where we found effects of father's ring colour these were assumed to be due to differential female investment in the eggs. Effects of foster father's ring colour are assumed to be due to maternal effects following hatching i.e. nestling provisioning.

Egg Size

Females mated to red ringed males in experiment 1 laid significantly larger eggs towards the end of the laying sequence than females mated to green ringed males (Fig. 1a). Rutstein *et al.* (2004b) also found this in a study examining resource allocation in zebra finches in relation to male ring colour. Similarly, an increase in the size of eggs laid for attractive males has been found in mallards (*Anas platyrhynchos*) (Cunningham and Russell 2000). We did not find this pattern in experiment 2 where egg size increased with laying order (Fig. 1c) but there were no differences in egg size in relation to male ring colour.

Egg production is costly for birds (Monaghan and Nager 1997) and this has been demonstrated for zebra finches in terms of energy expenditure (Williams and Ames 2004) and acquiring nutrients for egg formation (Houston *et al.* 1995; Williams and Martyniuk 2000). Increasing egg size in relation to male ring colour may therefore represent an additional costly investment. The consequences of increased egg production have been evaluated for birds laying more eggs rather than laying larger eggs. It has been found that increasing clutch size may be detrimental to the condition and survival of the laying female (Nager *et al.* 2001; Veasey *et al.* 2001).

Zebra finch chicks hatch asynchronously and are fed and grow rapidly from hatching resulting in an age and size hierarchy within the brood. In asynchronously hatching species later hatched chicks are more vulnerable, show a slower growth rate (Lago *et al.* 2000; Johnson *et al.* 2003) and higher mortality (Graves *et al.* 1984; Mock *et al.* 1990), as they

must compete with older, larger siblings for food provided by the parents. By increasing the size of later laid eggs when mated to an attractive male, females may enhance their reproductive success as a positive relationship has been found between egg size and chick growth and survival, particularly in the first few days after hatching when mortality is highest (Williams 1994; Christians 2002). This relationship has been established for zebra finches. Rutstein *et al.* (2004a) found that egg mass was a significant predictor of post hatching mortality.

A recent study by Rutkowska and Cichon (2005) experimentally manipulated the clutches of zebra finch eggs to hatch synchronously and asynchronously. Egg size was found to have a significant effect on chick survival in synchronously hatching broods but not in asynchronously hatching broods. This suggests that egg size counteracts the negative effects of hatching asynchrony.

Increasing egg size is advantageous for chicks as larger eggs produce larger hatchlings better able to maintain their body temperature (Rhymer 1988) and with greater reserves of residual yolk. Additional benefits of egg size include the decreased surface to area volume ratio which results in larger eggs taking longer to cool than smaller eggs (O'Connor 1979) and allows them to lose water through evaporation at a lower rate (Drent 1970; Carey *et al.* 1983).

In captivity zebra finch eggs within a clutch tend to hatch one each day producing a brood with very marked size differences. In the wild differences in egg size with laying order may be more important as here hatching is less asynchronous occurring usually over 2 days (Zann 1996). The increased size of the youngest chicks hatching from larger eggs may result in them being of similar size and competitive ability to chicks which have hatched the previous day.

Clutch Size

We found no difference in the number of eggs laid for red and green ringed males. Zann (1994) found that in the wild, females mated to red ringed males laid an extra clutch of eggs during their lifespan, but that clutch size did not differ between females paired to red or green ringed males. Balzer and Williams (1998) examined clutch size in relation to mate attractiveness in the zebra finch using a cross over design where females were paired sequentially with both attractive and unattractive males. When paired to attractive males, females laid significantly larger clutches than when paired to unattractive males although this effect was weak. Our experimental design differed in that females laid only one clutch with

either a red or a green ringed male. This may account for our failure to detect the differences in clutch size with mate attractiveness found by Balzer and Williams.

The effect of dietary supplementation in increasing both egg and clutch size in zebra finches is well documented (e.g. Williams 1996a; Williams and Miller 2003; Rutstein *et al.* 2004a). However, when maintained on a stable diet clutch size appears to be a relatively inflexible trait varying widely between individuals but showing high repeatability within individual females (Williams 1996b).

Yolk Hormones

We found no difference in the concentration of testosterone or DHT with male ring colour. This result was unexpected and would suggest that females are not differentially investing androgens in eggs laid for red ringed males. However, we sampled only the second laid egg from each clutch, which presents us with an incomplete picture of the pattern of allocation both within and between clutches laid by different females. A recent study by von Engelhardt *et al.* (2004) on zebra finches examined total androgen concentrations in first and fourth laid eggs of clutches laid for attractive and unattractive males. Females mated to attractive males deposited significantly more androgens in fourth laid eggs than females mated to unattractive males. No differences were found between the two groups for first laid eggs. This suggests that later laid eggs might be a more useful indicator of inter clutch differences in yolk androgen.

Gil *et al.* (1999) showed that females laid eggs with greater androgen levels when paired to a red ringed male. Their experiment used a cross over design where females were paired sequentially with both a red and green ringed male. This design allows the variation between females to be accounted for and showed that individual females invested higher levels of androgens in eggs laid for red ringed males than in those laid for green ringed males. In our experiments females were paired once only with either a red or green ringed male. This did not allow us to eliminate between female variation in androgen levels. Several authors have documented that the variation in yolk testosterone levels between clutches laid by different females is greater than the variation within clutches laid by the same female (e.g. Reed and Vleck 2001). Our females may have laid eggs containing higher levels of androgens when mated to red ringed males than they would when mated to green ringed males but due to high between female variation in yolk androgen levels we were unable to detect this. Whilst we have not shown that testosterone levels are higher in eggs laid for red ringed males we cannot eliminate the possibility that maternal effects seen here are due to yolk testosterone.

Begging

Complex differences in time spent begging in relation to both mate attractiveness and experimental replicate were found. These have already been discussed above.

Growth

Asynchronous hatching creates an age and size hierarchy within broods. Smaller, later hatched chicks often grow more slowly and fledge at a lower mass and body size than their earlier hatched siblings (e.g. Lago *et al.* 2000; Johnson *et al.* 2003). This expected pattern of decreasing growth rate with hatching order is apparent for weight gain in experiments 1 and 2 together (Fig. 6c) and tarsus gain for experiment 2 (Fig. 5c). In experiment 1 tarsus increase (Fig. 5a) shows the expected decline with laying order for chicks with green ringed fathers but is similar across laying order for chicks with red ringed fathers. This shows a greater than expected growth rate for later hatched chicks with red ringed fathers which appears to compensate for the effects of hatching asynchrony on growth rate. It is unclear whether this increase may be due to increased food provisioning by the parents due to the higher begging rate found in these chicks or to the effects of some constituent of egg composition.

Chicks hatching from testosterone manipulated eggs of European starlings (Pilz *et al.* 2004), canaries (Schwabl 1996b) and black-headed gulls (Eising *et al.* 2001) showed an increased rate of growth over control chicks in the period of early development following hatching. This increased growth was proposed by both Schwabl and Eising to be due to chicks hatching from testosterone treated eggs begging more and receiving a greater share of food. We found that chicks with red ringed fathers begged more in experiment 1 which may have led to greater food acquisition. However, we found no increase in mass gain for chicks with red ringed fathers, which might be expected if chicks were being fed more. Pilz *et al.* (2004) found an increase in chick growth in the first few days following hatching but no accompanying difference in begging behaviour. The increased tarsus growth rate found here might be due to differences in egg quality and possibly yolk androgen levels.

Weight gain for both experiments shows a trend for foster father's ring colour (Fig. 6a). Chicks with red ringed foster fathers had a greater weight gain suggesting that they were being provided with more food by the parents. Increased nestling provisioning of the chicks of red ringed males has been shown previously in zebra finches by Burley (1988b). However, in Burley's study the birds were housed in an aviary environment where the possibility of assortative mating could not be excluded. Therefore her results may have been due to good quality females, which are likely to provision their brood at a greater rate, pairing with

attractive males. A recent study on blue tits (*Parus caeruleus*) in the wild (Limbourg *et al.* 2004) experimentally manipulated the attractiveness of males within existing pairs that had already laid a clutch of eggs. The UV reflectance of the male's crown feathers, an indicator of sexual attractiveness and male viability, was reduced shortly before the eggs hatched. Females mated to experimental males reduced their nestling feeding effort in comparison to controls resulting in lower chick growth rates and potentially reduced offspring fitness.

3. Additional Results

Here any remaining results revealed by the statistical analysis that do not relate to either experimental differences or mate attractiveness are discussed.

Clutch Size

Clutch size showed a trend in relation to female mass. Heavier females laid larger clutches. Studies of dietary intake in relation to egg laying have also found this (Rutstein *et al* 2004a). It is assumed that females with greater body reserves are better able to lay a larger number of eggs.

Begging

Brood size had a significant effect on the time spent begging in experiment 2 (Fig. 4d). This supports the proposal that begging strategies can be learned (Kedar *et al.* 2000; Rodriguez-Girones *et al.* 2002) in relation to the composition of the brood and parental feeding strategies. Parents of larger broods must presumably provision the brood at a higher rate, making more visits to their nest with food than would a parent with fewer young. This increased rate of food delivery may reduce the amount of time individual chicks need to beg to ensure they are fed. Mathevon and Charrier (2004) found that the number of chick begging bouts in black-headed gulls decreased as brood size increased.

Growth

Brood size showed an effect on tarsus increase in experiment 1 such that chicks from larger broods had a greater tarsus increase (Fig. 5b). This result is surprising as most studies show a reduced growth rate for chicks from larger brood sizes due to sibling competition for food (De Kogel and Prijs 1996; Deerenberg *et al.* 1996; De Kogel 1997). In these studies brood size was manipulated to provide the experimenter with the desired differences in number of

offspring raised within each nest to meet the criteria of the study. In our experiment, although eggs were cross fostered between nests clutch sizes were maintained. It appears that good quality parents capable of laying and successfully incubating a larger clutch are also able to provision all the nestlings successfully. Poorer parents who had mortality amongst nestlings due to their inability to initiate feeding or provide adequate quantities of food subsequently had smaller broods of nestlings with lower growth rates.

Results Appendix

Egg Weight

Experiment 1

Variable	df	F	p
Laying Order	1, 128	62.07	<0.0001
Male Ring Colour	1, 27	0.63	0.4348
Female Mass	1, 128	3.69	0.0569
Laying Order*Male Ring Colour	1, 128	4.71	0.0318
Laying Order*Female Mass	1, 127	0.44	0.5101

Experiment 2

Variable	df	F	p
Laying Order	1, 116	11.5	0.0009
Male Ring Colour	1, 28	2.21	0.1486
Female Mass	1, 116	0.02	0.8793
Laying Order*Male Ring Colour	1, 114	0.35	0.5543
Laying Order*Female Mass	1, 115	2.98	0.0868

Experiments 1 and 2

Variable	df	F	p
Laying Order	1, 253	40	<0.0001
Male Ring Colour	1, 47	0.02	0.8763
Female Mass	1, 253	4.42	0.0365
Experiment Number	1, 253	1.12	0.2907
Laying Order*Male Ring Colour	1, 253	3.39	0.0666
Laying Order*Experiment Number	1, 253	7.36	0.0071

Clutch Size

Experiment 1

Variable	df	ss	F	p
Female Mass	1	1.947	1.23	0.2764
Male Ring Colour	1	0.02809	0.02	0.8968

Experiment 2

Variable	df	ss	F	p
Female Mass	1	0.1636	0.15	0.7042
Male Ring Colour	1	0.804	0.75	0.3950

Experiments 1 and 2

Variable	df	ss	F	p
Female Mass	1	4.4214	3.9	0.531
Male Ring Colour	1	0.07783	0.07	0.7959
Experiment Number	1	10.63	9.37	0.0033

Yolk Hormones

Experiment 1

Dihydrotestosterone

Variable	df	ss	F	p
Male Ring Colour	1	2111524	0.33	0.569
Female Mass	1	235642	0.04	0.8486
Clutch Size	1	19287	0.00	0.9564

Testosterone

Variable	df	ss	F	p
Male Ring Colour	1	87345130	2.88	0.1031
Female Mass	1	12329279	0.41	0.53
Clutch Size	1	1567967	0.05	0.8221

Experiment 2

Dihydrotestosterone

Variable	df	ss	F	p
Male Ring Colour	1	1048857	0.17	0.6871
Female Mass	1	6803472	1.08	0.3104
Clutch Size	1	1628	0.00	0.9873

Testosterone

Variable	df	ss	F	p
Male Ring Colour	1	11297	0.00	0.9883
Female Mass	1	20022594	0.39	0.5395
Clutch Size	1	642037	0.01	0.9121

Begging

Experiment 1

Variable	df	F	p
Father Ring Colour	1, 14	8.19	0.0126
Foster Father Ring Colour	1, 13	0.49	0.4979
Female Mass	1, 26	0.17	0.6865
Hatching Order	1, 25	0.06	0.8125
Chick Sex	1, 27	0.27	0.6073
Broodsize	1, 25	0.01	0.9061

Experiment 2

Variable	df	F	p
Father Ring Colour	1, 17	12.07	0.0029
Foster Father Ring Colour	1, 17	7.64	0.0133
Female Mass	1, 25	0.05	0.8186
Hatching Order	1, 26	0.34	0.5651
Chick Sex	1, 28	1.84	0.1862
Broodsize	1, 34	6.57	0.015

Experiments 1 and 2

Variable	df	F	p
Male Ring Colour	1, 29	0.81	0.3747
Foster Father Ring Colour	1, 29	10.2	0.0034
Female Mass	1, 56	0.23	0.6346
Hatching Order	1, 53	0.01	0.9277
Chick Sex	1, 57	0.71	0.4020
Broodsize	1, 64	3.37	0.0709
Experiment Number	1, 64	0.74	0.3925
Father Ring Colour*Experiment No.	1, 64	30.29	<0.0001
Fost. Father Ring Colour* Exp. No.	1, 64	5.56	0.0214

Growth

Weight Gain

Experiment 1

Variable	df	F	p
Father Ring Colour	1, 13	1.41	0.2567
Foster Father Ring Colour	1, 12	1.28	0.2795
Female Mass	1, 25	0.32	0.5744
Hatching Order	1, 23	0.05	0.8208
Chick Sex	1, 26	2.07	0.1621
Broodsize	1, 26	0.52	0.4772

Experiment 2

Variable	df	F	p
Father Ring Colour	1, 14	0.06	0.8106
Foster Father Ring Colour	1, 15	1.75	0.2062
Female Mass	1, 27	0.73	0.4019
Hatching Order	1, 22	1.27	0.2728
Chick Sex	1, 23	2.47	0.13
Broodsize	1, 21	0.13	0.723

Experiments 1 and 2

Variable	df	F	p
Father Ring Colour	1, 31	1.09	0.3048
Foster Father Ring Colour	1, 32	3.66	0.0647
Female Mass	1, 60	0.9	0.3453
Hatching Order	1, 60	4.71	0.034
Chick Sex	1, 55	0.89	0.3498
Broodsize	1, 55	0.01	0.9153
Experiment Number	1, 60	13.8	0.0004

Tarsus Increase

Experiment 1

Variable	df	F	p
Father Ring Colour	1, 12	2.92	0.113
Foster Father Ring Colour	1, 11	0.31	0.5887
Female Mass	1, 27	1.46	0.2377
Laying Order	1, 26	2.83	0.1047
Chick Sex	1, 24	0.61	0.4423
Broodsize	1, 26	4.73	0.0389
Male Ring Colour*Laying Order	1, 26	9.39	0.005

Experiment 2

Variable	df	F	p
Father Ring Colour	1, 15	0.19	0.6675
Foster Father Ring Colour	1, 17	0.52	0.4794
Female Mass	1, 28	0.02	0.8941
Laying Order	1, 28	8.14	0.008
Chick Sex	1, 24	0.09	0.7659
Broodsize	1, 28	0.21	0.6533

Experiments 1 and 2

Variable	df	F	p
Father Ring Colour	1, 31	4.42	0.0438
Foster Father Ring Colour	1, 29	0.61	0.4395
Female Mass	1, 57	0.42	0.5194
Hatching Order	1, 27	4.05	0.0488
Chick Sex	1, 53	0.18	0.6754
Broodsize	1, 57	1.76	0.1898
Experiment Number	1, 57	35.02	<0.0001
Male Ring Colour*Hatching Order	1, 57	8.92	0.0042

Survival

Experiment 1

Variable	df	F	p
Father Ring Colour	1, 18	1.33	0.2636
Foster Father Ring Colour	1, 17	0.02	0.8932
Female Mass	1, 38	1.12	0.2971
Laying Order	1, 40	0.00	0.9466
Hatching Weight	1, 40	1.14	0.2919
Broodsize	1, 39	1.15	0.2894

Experiment 2

Variable	df	F	p
Father Ring Colour	1, 21	1.95	0.1774
Foster Father Ring Colour	1, 20	0.95	0.3426
Female Mass	1, 42	0.79	0.3801
Laying Order	1, 42	1.26	0.2675
Hatching Weight	1, 41	2.38	0.1303
Broodsize	1, 43	0.59	0.4471

Experiments 1 and 2

Variable	df	F	p
Father Ring Colour	1, 43	2.18	0.1475
Foster Father Ring Colour	1, 41	0.11	0.7363
Female Mass	1, 89	0.62	0.4324
Laying Order	1, 90	0.36	0.5493
Hatching Weight	1, 88	0.52	0.4741
Experiment Number	1, 91	1.64	0.2038
Broodsize	1, 40	1.64	0.208

3

Mate Attractiveness, Maternal Effects and Yolk Testosterone

Part Two

Adult Characteristics

In the previous chapter I investigated egg composition as a maternal effect in relation to mate attractiveness in the zebra finch. The study examined how changes in egg composition influence chick development from hatching to independence. Particular emphasis was placed on yolk androgen concentration as the potential mediator of maternal effects due to egg composition.

The majority of experimental studies on the effects of increased yolk androgens on development have examined chick development until fledging. Few investigations have looked into any effects carried into adulthood. To date studies have only examined the effects on adult morphology, attractiveness and dominance. In this chapter the investigation continues by looking at a range of potential effects carried into adulthood, and again the focus will be on yolk androgens.

Effects of Yolk Androgens on Adult Morphology

Strasser and Schwabl (2004) experimentally increased yolk testosterone levels in house sparrow eggs (*Passer domesticus*) by injection. As adults, males hatching from testosterone treated eggs had significantly larger patches of black throat feathers, known as 'badges', than control males. Badge size in male house sparrows appears to be correlated with their social dominance (Hein *et al.* 2003) and attractiveness to females (Veiga 1993).

Effects of Yolk Androgens on Attractiveness

An experimental study on zebra finches (*Taeniopygia guttata*) also increased yolk testosterone levels through injection. Here it was found that both male and female offspring hatching from experimental eggs were more attractive than controls in mate choice trials (von Engelhardt 2004). The reasons for this are unclear, as there did not appear to be any phenotypic differences between birds from the two groups. This may suggest that behavioural differences were important.

Effects of Yolk Androgens on Dominance

Dominance has been examined in two studies using similar methods (Schwabl 1993; Strasser and Schwabl 2004). Birds were deprived of food for a short time then presented with a single food source. Dominance was measured as latency to feed, time spent feeding and through observation of aggressive interactions. Schwabl (1993) used groups of 3 canary siblings (*Serinus canaria*) and found a correlation between social rank and the level of testosterone in

the eggs from which the chicks had hatched. Testosterone levels had been determined by yolk sampling.

Strasser and Schwabl (2004) tested pairs of 5-month-old house sparrow chicks closely matched to be physically similar except that one bird had hatched from a testosterone treated egg and the other was a control. Testosterone treated chicks were first to approach the food source and spent significantly longer feeding at the beginning of each trial than control chicks.

Aims

Attractiveness in zebra finches can be manipulated using coloured leg rings. Males wearing red leg rings are perceived as attractive to females, whereas those wearing green leg rings are unattractive (Burley *et al.* 1982; see Chapter 1 for discussion). Gil *et al.* (1999) found that female zebra finches mated to red ringed males deposit significantly higher levels of testosterone in their eggs than those mated to green ringed males. Our intention was to examine the effects of increased yolk testosterone due to mate attractiveness on adult characteristics in the zebra finch. This was achieved by comparing offspring hatching from eggs laid for red ringed and green ringed males.

We looked at final body size in terms of weight and tarsus length, rate of maturity, dominance, the attractiveness of male offspring and the reproductive characteristics of female offspring. Chicks hatching from eggs laid for red ringed males were expected to show a faster rate of maturity, greater adult size and to be more dominant than chicks hatching from eggs laid for green ringed males. It was further predicted that for chicks with red ringed fathers, male offspring would be more attractive themselves and female offspring would show enhanced reproductive characteristics in terms of clutch or egg size.

For zebra finches measuring the rate of maturity may be particularly significant as they are one of only five bird species known to show precocial breeding (Zann 1996). This is where, due to their extended breeding season, chicks can mature and reproduce in the same season in which they hatched potentially greatly enhancing their reproductive success (Zann 1996).

Methods

An outline of the experimental design and statistical methods are covered in the preceding chapter (chapter 2). For final size, experiments 1 and 2 were analysed both separately and together, as in chapter 2. For all other traits experiments 1 and 2 were not analysed separately due to small sample sizes. Here analysis was of both experiments together although to

establish that differences did not exist between experimental replicates, experiment number was included as an explanatory variable. In all cases it was removed early in the analysis.

Final adult size, rate of maturity and attractiveness of male offspring were analysed using mixed general linear models. Dominance was analysed using mixed generalized linear models, as the data was binomial.

Final Size

Final measurements of tarsus and weight were taken once the birds had acquired full adult plumage at about 100 days old.

Maturity

Juvenile zebra finches have black beaks and grey plumage. Between the ages of about 35 and 60 days their sexually dimorphic plumage starts to appear and beak colour changes via pink to red in males and orange in females (De Kogel 1997). Out of a total of 107 chicks successfully reared to fledging, eight chicks were found to be white morphs and eleven were fawns. These birds were excluded from the maturity investigation, as it was not known whether different colour morphs mature at different rates.

The beaks of all wild type juveniles were photographed at 50 days of age using a Fuji finepix digital camera. For each bird 3 photographs were taken of the beak from above, left and right. These pictures were analysed using the computer programme 'Sigma Scan' which accurately measures areas from digital photographs. From this we determined the proportion of black remaining on the upper mandible as a measure of maturation. The results from the 3 pictures were pooled to give a total mean score of the proportion of black on the upper mandible of each bird. A smaller score indicated a smaller area of black and a more mature bird. As the results were proportions they were arcsine transformed before analysis.

Dominance

Zebra finches in the wild are known to compete over resources such as food, mates, roost sites etc. (Zann 1996). We manipulated the value of food as a resource by depriving birds of food for a period of 4 hours. After hatching each brood was kept isolated. This meant they would be naïve to competition from birds outside their family group reducing the possibility of learning. Birds were tested in pairs matched for sex, colour morph, hatching order and laying order with each bird only used in one trial. Therefore, within each pair the birds were as

similar as possible except that one bird had a red ringed father and the other had a green ringed father.

White morph males and females differ only in their beak colour. As this is an unreliable indicator of sex, they were sexed using molecular techniques (Rutstein *et al.* 2004a) following extraction of DNA from feather samples (Taberlet and Bouvet 1991). A description of these techniques is given below.

A large cage measuring 228 cm x 40 cm x 40 cm that could be divided into 3 sections with partitions was used for the dominance trials. This allowed the birds to be kept visually isolated at either end of the cage during the food deprivation period. One bird was placed at either end of the triple cage and left for a period of 4 hours with water but no food. When the partitions were removed they had access to the central section of the cage where a seed hopper was set up to allow only one bird to feed at a time. Once introduced to the central section the birds were videoed for a period of 20 minutes. Following this they were returned to their original cages where food and water was freely available.

I intended to measure dominance as the amount of time spent at the food either feeding or preventing the other bird from gaining access. However, during the trials it became clear that there was considerable variation in how the birds displayed their dominance. Some birds controlled access to the feeder whereas others showed little interest in the food, although it was apparent from their behaviour that they were dominant. As a result additional behavioural interactions were considered in assessing dominance.

Dominance was determined from the account of zebra finch behaviour described in Zann 1996 and Morris 1954. A bird was considered dominant if it displayed the following aggressive behaviours: initiating bill fencing, plucking feathers from opponent, adopting a horizontal posture when facing opponent, chasing an opponent either on the perch or on the cage floor. Submissive birds may adopt a 'fluffed-up' appearance, retreat from an attacker and often fly to and from the perch when intimidated by a more dominant bird. If it was unclear which bird was dominant, no decision was given for that trial and it was not included in the final result.

DNA Extraction from Feathers

DNA from feathers was extracted as follows: fragments of feather samples were added to 400µl cell lysis solution (0.1M EDTA, 0.2M Tris pH 8.5, 1% SDS, 0.1M NaCl, 10mg/ml DTT). Following mixing 0.5 mg/ml proteinase K was added before incubation at 55°C

overnight. To remove DNA from the digested tissue 200µl phenol was added to each sample, mixed and centrifuged at high speed for 8 minutes. The supernatant was retained and 100µl each of phenol and chloroform were added, mixed and spun for a further 8 minutes and the supernatant retained. A further 200µl of chloroform was added, mixed and spun for 8 minutes and the supernatant retained. This was spun for 2 minutes before being decanted into a clean tube. Precipitation of DNA was achieved by adding 40µl 3M Sodium acetate at pH 5.2 and 1ml cold 100% ethanol. This was mixed by inversion then left on the bench for 10-15 minutes. Samples were centrifuged for 10 minutes and the supernatant poured off. The pellet was washed with 600µl 70% ethanol and centrifuged for 5 minutes. The ethanol was poured off and the washing step repeated. Samples were left to dry for 20 minutes before the pellet was re-suspended in 25µl MilliQ water.

Molecular Sexing

A fragment of the W-linked CHD gene (CHD-W) in females, and its Z-linked homologue (CHD-Z), present in both sexes was amplified by PCR using primers and methodology modified from Griffiths *et al.* (1998). PCRs comprised 50-250ng of genomic DNA, 0.2µl 8mM dNTP, 0.2µl each of 50mM primers ZF1 (5' TGA GAA ACT GTG CAA AAC AGG 3') and ZF2 (5' TTT TCT CGA GGA ATA GTT CG 3'), 0.5 units of *Taq* polymerase (Bioline), 0.6µl 50nM $MgCl_2$ 1µl Bioline 10xNH_4 reaction buffer, in a total volume of 10µl. Reactions were performed with the following thermal profile: initial denaturation at 94°C for 30s, 56°C for 45s, 72°C for 45s, and finally 72°C for 5 minutes. PCR products were separated on 3% agarose gels at 50V and visualised with ethidium bromide. Birds were sexed according to the presence of the PCR products of CHD-Z (350 base pairs; both sexes) and CHD-W (384 base pairs; females only).

Offspring Clutch Size

Female offspring were allowed to breed under the conditions described in chapter 1. Females were paired with males whose attractiveness had not been manipulated i.e. they wore a single orange identification leg ring only. All eggs were collected on the day of laying, weighed, measured, and replaced with dummy eggs. Females were not allowed to raise chicks.

Attractiveness of Male Offspring

Attractiveness of male offspring was measured through female choice using a choice chamber. The choice chamber was built to a design shown in Figure 1. A female with breeding experience was placed in the large compartment (A). Males were placed in the two smaller compartments (B and C). Females were considered to have chosen a male when they sat on the perch nearest to him. Time spent on the perch at the rear of the cage or on the floor was not included. Each pair of males was matched as closely as possible for size, hatching order and laying order and differed only in the attractiveness of their father. Within each pair one male had a red ringed father and one a green ringed father. All birds had access to food and water throughout the experiment; this was placed in a neutral zone so as not to influence the result.

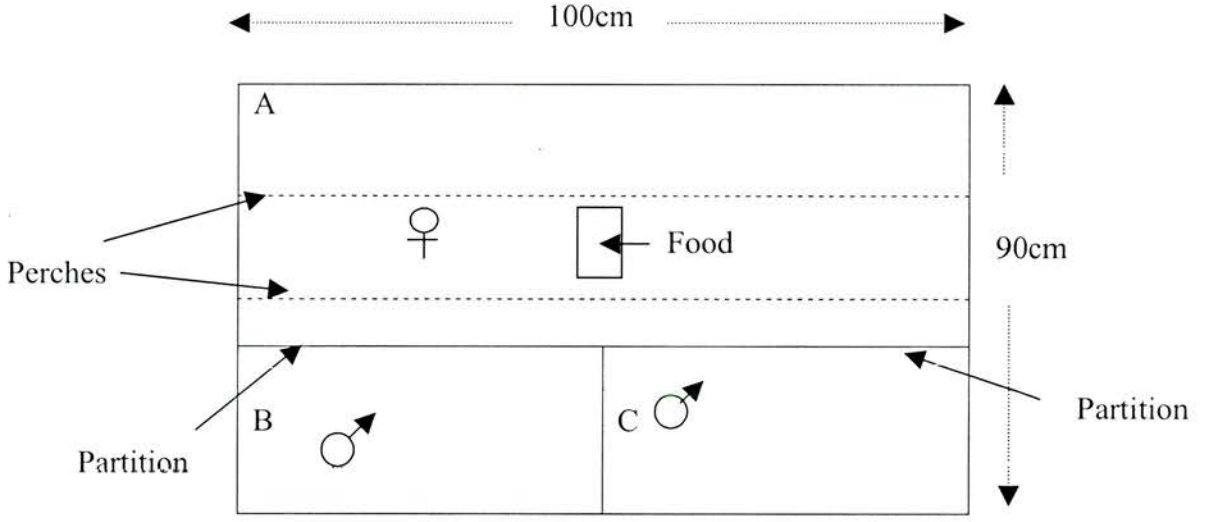


Figure 1. Choice chamber to study attractiveness of male offspring.

Birds were placed in the choice chamber at the start of the experiment with partitions in place between compartment A and compartments B and C. They were left to settle for 20 minutes after which the partitions were removed. The 20-minute trial began when it was apparent that the female had seen both males and was therefore in a position to make a choice between them. The time spent by the female on the perches nearest the males was recorded using stopwatches. Birds were observed using a Sony Handycam video camera. The camera filmed the birds and this was played directly onto a monitor placed behind a screen and watched by an observer. At the end of the trial the partitions were replaced. The males were then swapped between compartments B and C and allowed to settle for a further 20 minutes. The trial was

then repeated. This change over of the males allowed for any potential side bias shown by the females.

Side bias was eliminated using the methods of Blount *et al.* (2003). This involved adjusting the actual time spent by the female on each side of the choice chamber in relation to that which would be expected if no side bias existed as follows: If no side bias existed females would be expected to spend 50% of their total time on each side of the cage across the two trials, i.e. in the perfect situation where no side bias exists and an attractive male is placed on the left of the choice chamber, the female will spend 100% of her time with this male and 0% of her time with the unattractive male (see below). The males are then swapped, the attractive male is now on the right of the choice chamber and the female spends 100% of her time on the right. The total for each side is then divided to give the expected percentage time for each side across both trials.

	Percentage of time		Percentage for each trial	
	Left	Right	Left	Right
1st Trial	100%	0%	50%	50%
2nd Trial	0%	100%		

If the female spends more than 50% of her time on one side it suggests she has a preference for that side. Therefore, to correct for side bias during the actual trials the percentage time spent by the female on each side was calculated as above. If the value was greater than 50% for a side then the difference was subtracted from the proportion of time spent by the female on that side for each trial. Similarly if the value was less then 50% then the difference was added to the percentage time spent on each side. An example is given below that may help explain this method.

In the following example a male with a red ringed father was placed in the right hand section of the choice chamber first, then in the second trial was swapped to the left hand section. The female shows a bias for the right hand side of the cage.

	Time in Seconds		Percentage of Time	
	Left	Right	Left	Right
1st Trial	65	273	19	81
2nd Trial	306	172	64	36

Total % time for both Trials		Total % for single trial	
Left	Right	Left	Right
83	117	41.5	58.5

If no side bias exists then time spent on each side for a single trial would be expected to be 50%. Therefore add or subtract difference to give adjusted time.

Adjusted % Time

	Left	Right
1st Trial	27.5	72.5
2nd Trial	72.5	27.5

As the data was in the form of proportions of time spent by the female with each male it was arcsine transformed before analysis. Statistical analysis used the data for preferred males only. As males were tested in pairs and the data given, as proportions then including both results in the data set would introduce bias since the results from the males within each pair is related. This violates one of the assumptions of general linear models.

Results

Final Size

Tarsus

Experiment 1

Variables used were fathers ring colour, foster fathers ring colour, female mass, laying order, hatching order, chick sex and broodsize. Sample size was 44 chicks.

Father's ring colour and chick sex showed a highly significant interaction ($F_{1, 27}$ =16.14, P=0.0004) (Fig. 1a). Female chicks with red ringed fathers were bigger than females with green ringed fathers ($F_{1, 12}$=23.81, P=0.0004). Males showed little difference in final tarsus length with father's ring colour ($F_{1, 9}$=0.15, P=0.7099). Laying order showed no effect on final tarsus length in experiment 1 ($F_{1, 26}$=0.24, P=0.6319).

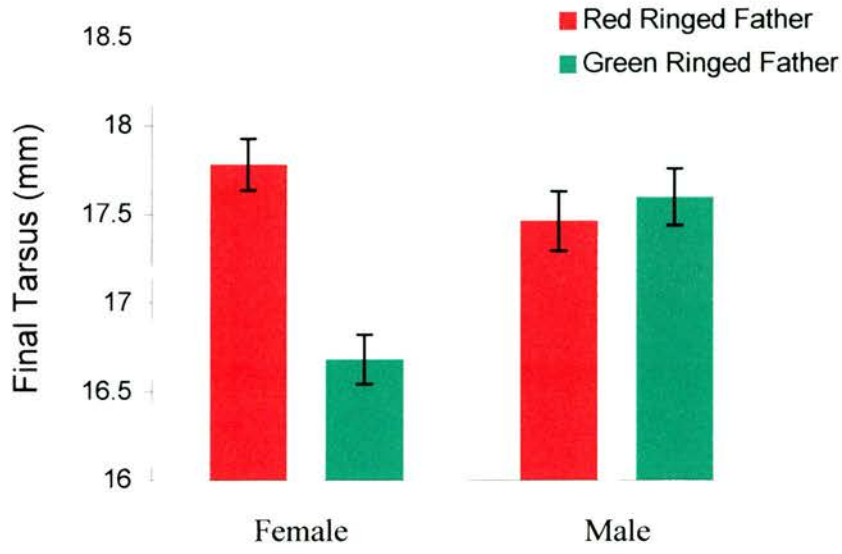


Figure 1a. Fitted mean final tarsus length (mm ± s.e.) with chick sex and fathers ring colour for experiment 1.

Experiment 2

Variables used were fathers ring colour, foster fathers ring colour, female mass, laying order, hatching order, chick sex and broodsize. Sample size was 54 chicks.

There was a significant interaction between father's ring colour and chick sex in experiment 2 ($F_{1,29}$ =19.92, P=0.0001) (Fig.1b). Female chicks with red ringed fathers were larger than those with green ringed fathers.

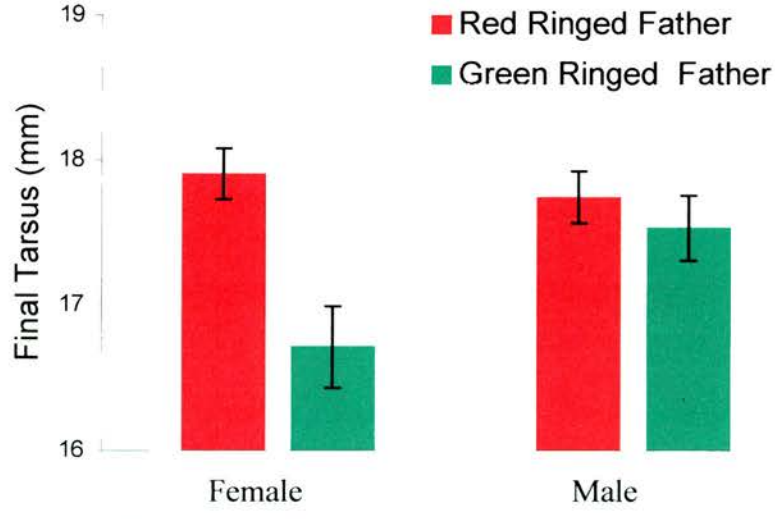


Figure 1b. Fitted mean final tarsus length (mm ± s.e.) with chick sex and fathers ring colour for experiment 2.

In experiment 2 final tarsus length decreased significantly with hatching order ($F_{1,29}$ =28.61, P=<0.0001) (Fig. 1c).

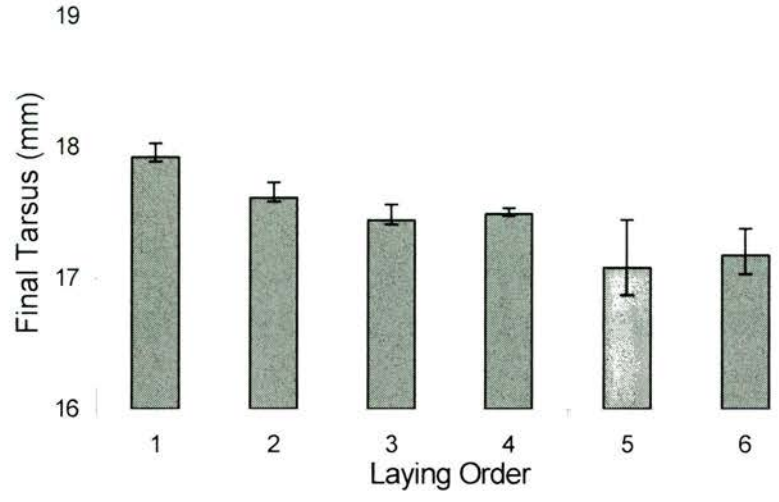


Figure 1c. Fitted mean final tarsus length (mm ± s.e.) with hatching order for experiment 2.

Experiments 1 and 2 Together

Variables used were fathers ring colour, foster fathers ring colour, female mass, laying order, hatching order, experiment number, chick sex and broodsize. Sample size was 101.

Chick sex and father's ring colour showed a significant interaction for final tarsus length for experiments 1 and 2 together ($F_{1, 56}$ =13.96, P=0.0004) (Fig. 1d). Female chicks with red ringed fathers were larger than those with green ringed fathers ($F_{1, 30}$=16.55, P=0.0003). Males showed little difference in final tarsus length with father's ring colour ($F_{1, 26}$=2.39, P=0.1339).

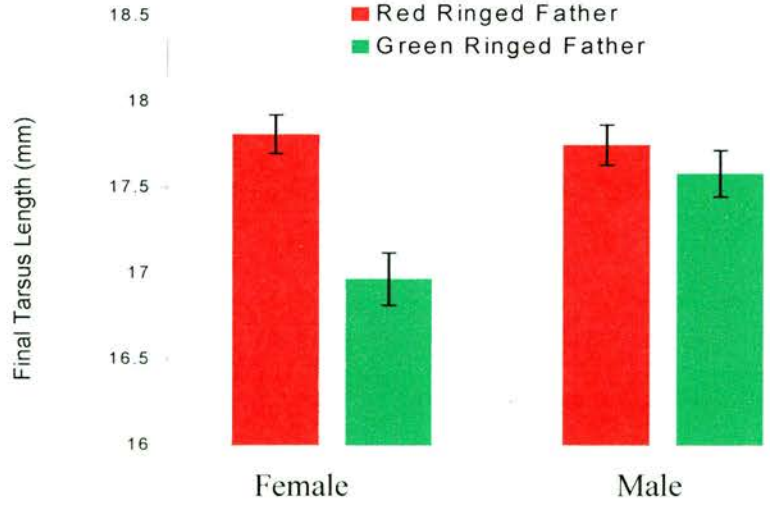


Figure 1d Fitted mean final tarsus length (mm ± s.e.) with fathers ring colour and offspring sex for experiments 1 and 2 together.

Final tarsus decreased significantly with laying order ($F_{1, 56}$ =16.59, P=0.0001) (Fig. 1e).

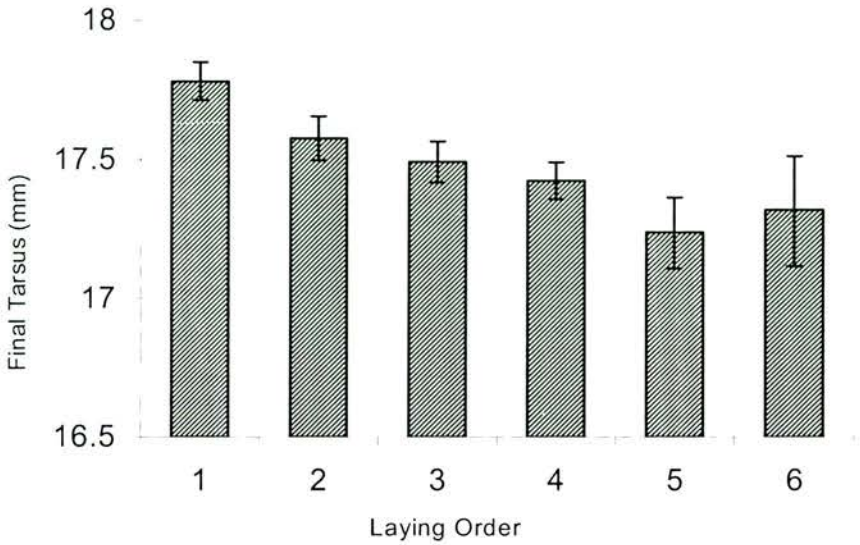


Figure 1e. Fitted mean final tarsus (mm ± s.e.) with laying order for experiments 1 and 2.

Weight

Experiment 1

Variables used were fathers ring colour, foster fathers ring colour, female mass, laying order, hatching order, chick sex and broodsize. Sample size was 44 chicks.

Chick sex showed a non-significant trend with female chicks having a greater final weight than males ($F_{1, 28}$ =4.01, P=0.0551) (Fig. 2a). Hatching order had no significant effect on final weight ($F_{1, 27}$=0.22, P=0.6417).

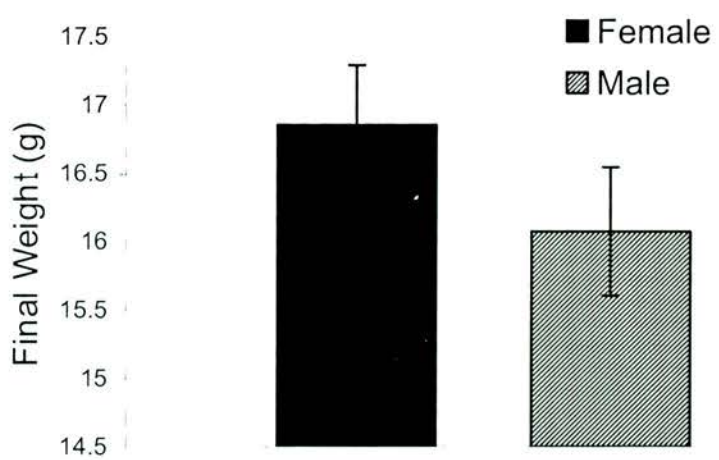


Figure 2a. Fitted mean final weight (g ± s.e.) with chick sex in experiment 1.

Experiment 2

Variables used were fathers ring colour, foster fathers ring colour, female mass, laying order, hatching order, chick sex and broodsize. Sample size was 57 chicks.

Final weight showed a significant decrease with hatching order ($F_{1,\ 34}$ =13.97, P=0.0007) (Fig. 2b). Chick sex had no effect ($F_{1,\ 30}$=0.06, P=0.8062).

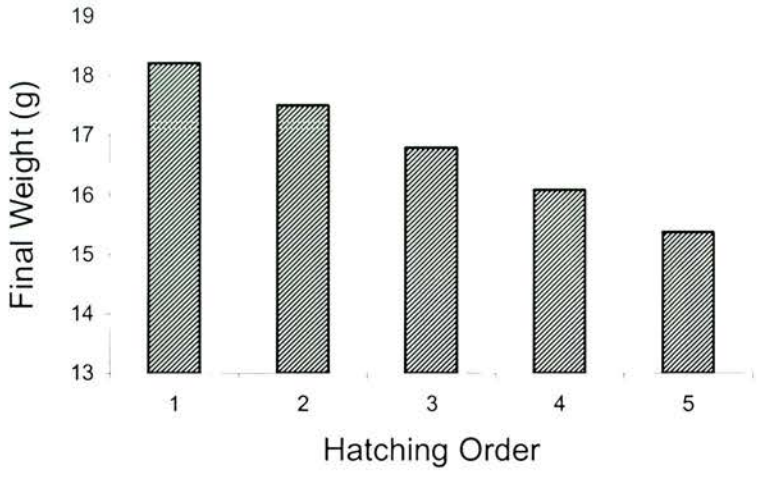


Figure 2b. Fitted mean final weight (g.) with hatching order for experiment 2.

Experiments 1 and 2 Together

Variables used were fathers ring colour, foster fathers ring colour, female mass, laying order, hatching order, experiment number, chick sex and broodsize. Sample size was 102 chicks.

Final weight showed a significant decrease with hatching order ($F_{1,\ 63}$ =12.06, P=0.0009) (Fig. 2c). There was no effect of chick sex ($F_{1,\ 56}$=0.27, P=0.6041).

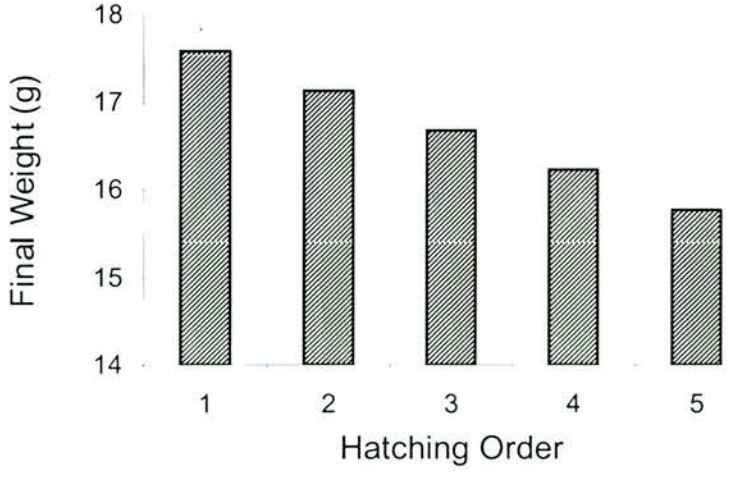


Figure 2c. Fitted mean final weight (g) with hatching order for experiments 1 and 2.

Dominance

Experiments 1 and 2 Together

Variables used were fathers ring colour, foster fathers ring colour, female mass, laying order, hatching order, chick sex and experiment number.

There was no effect of any of the variables examined on dominance.

Rate of Maturity

Experiments 1 and 2 Together

Variables used were fathers ring colour, foster fathers ring colour, female mass, laying order, hatching order, chick sex and experiment number. Sample size was 53 chicks.

The rate at which chicks matured, measured as the proportion of red on the beak at 50 days old, showed no relationship with any of the variables examined.

Female Offspring Clutch Size

Experiment 1 and 2 Together

Variables used were fathers ring colour, foster fathers ring colour, female mass, laying order, hatching order, experiment number, offspring final weight, offspring final tarsus length and broodsize. Sample size was 28 clutches.

None of the variables examined showed any significant effect on offspring clutch size.

Female Offspring Egg Mass

Experiments 1 and 2 Together

Variables used were fathers ring colour, foster fathers ring colour, female mass, laying order, hatching order, experiment number, offspring final weight, offspring final tarsus length and broodsize. Sample size was 140 eggs.

Father's ring colour had a significant effect on the mass of eggs laid by female offspring ($F_{1, 19} = 8.48$, P=0.0089)(Fig. 3a). Females with red ringed fathers laid significantly heavier eggs.

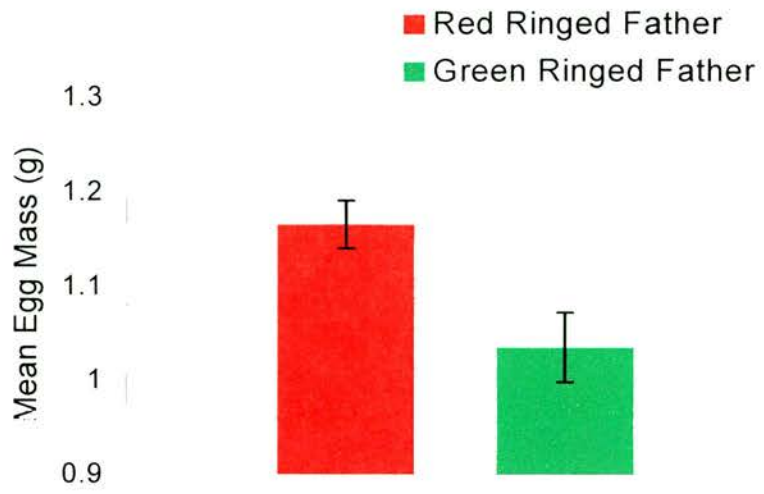


Figure 3a. Fitted mean offspring egg mass (g ± s.e.) with fathers ring colour for experiments 1 and 2.

Egg mass increased with laying order ($F_{1, 117}$=5.93, P=0.0164) (Fig. 3b).

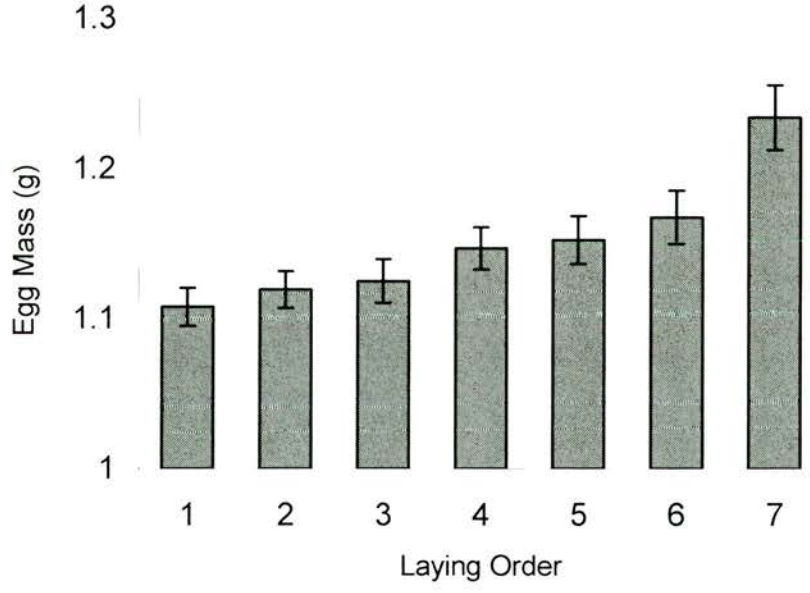


Figure 3b. Fitted mean offspring egg mass (g ± s.e.) and laying order for experiments 1 and 2.

Both female offspring mass ($F_{1, 117} = 15.45$, P=0.0001) (Fig. 3c) and tarsus length ($F_{1, 113} = 5.01$, P=0.0272) (Fig. 3d) had a significant effect on egg mass. Egg mass increased with both offspring tarsus length and offspring mass.

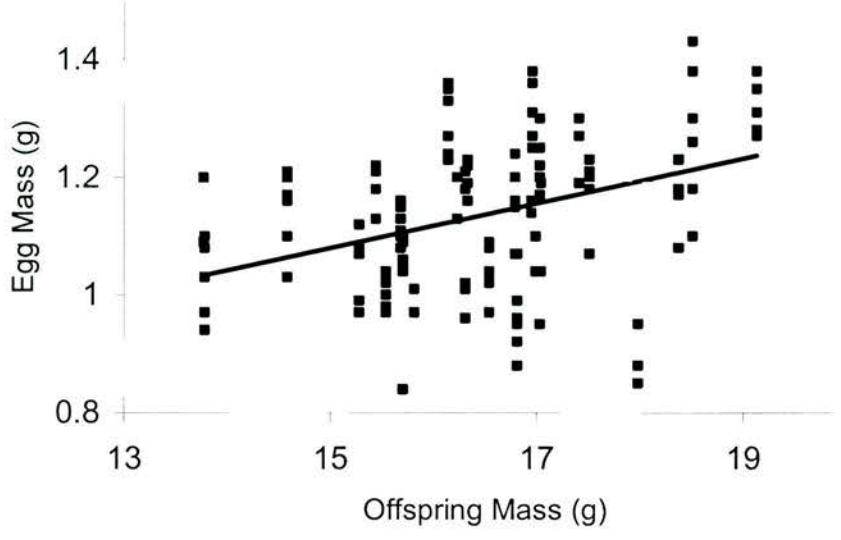


Figure 3c. Offspring egg mass (g) with offspring mass for experiments 1 and 2.

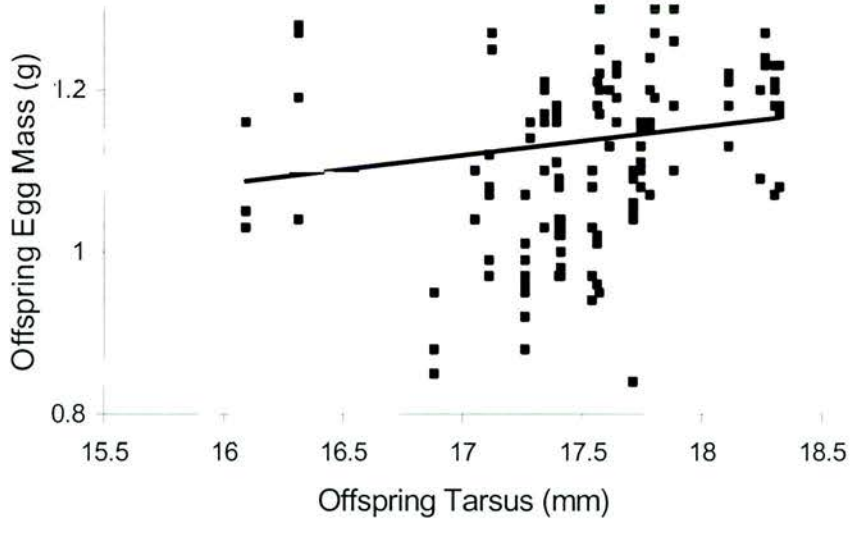


Figure 3d. Offspring tarsus (mm) and offspring egg mass (g) for experiments 1 and 2.

Attractiveness of Male Offspring

Experiments 1 and 2 Together

Variables used were fathers ring colour, female mass, laying order, hatching order, difference in weight between bird pairs and difference in tarsus length. Sample size was 16 pairs of males.

Father's ring colour showed a non-significant trend in relation to the attractiveness of his sons ($F_{1, 12.4}$=4.26, P=0.0606). Chicks with red ringed fathers tended to be more attractive themselves (fig. 4).

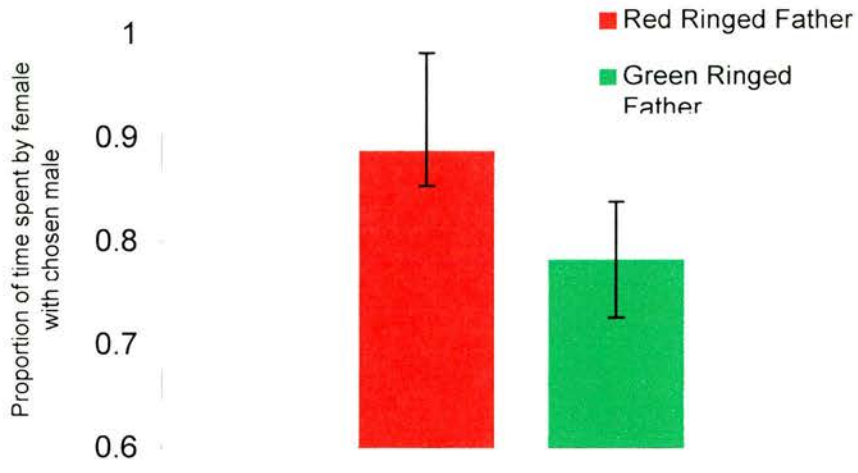


Figure 4. Fitted mean proportion of time spent with chosen male (± s.e.) for both experiments 1 and 2.

Discussion

As in the previous chapter the results will be divided into 3 sections.

1. Differences between Experiments

There were no significant differences between experiments for any of the traits examined. Experiment number was entered into the statistical models as an explanatory variable but was removed early in the analysis.

2. Effects of Male Ring Colour

Final Size

Tarsus

Previous studies on the effects of testosterone on chick growth have recorded growth rate up to the time of fledging (Schwabl 1996b; Eising *et al.* 2001; Pilz *et al.* 2004). No differences were found in weight or body size at fledging, but any potential effects on final adult size were not considered. Juvenile zebra finches fledge at 16 to 18 days old but do not attain final body size until around 30 days old and adult weight at about 50 days (Zann 1996).

Final tarsus length in experiments 1 and 2, when analysed both separately and together, showed a highly significant interaction between father's ring colour and offspring sex. Female offspring with red ringed fathers were larger than females with green ringed fathers (Figs. 1a, 1b and 1d). No differences were apparent between males in relation to father's ring colour.

Body mass and size in female birds has been found to explain up to 20% of the variation in the size of eggs that they lay (Christians 2002). Studies of zebra finches have shown body mass to be a predictor of egg size (Haywood and Perrins 1992; Williams 1996b). We found this as a trend in experiment 1 (Fig. 1b, Chapter 2) and it was significant when experiments 1 and 2 were analysed together (Fig. 1e, Chapter 2). The relationship between tarsus length, as a measure of body size, and egg size is less well studied. Given that tarsus length and body mass are correlated it could be predicted that tarsus length would also influence egg size. Analysis of tarsus length and egg size in experiment 2 showed that tarsus length had a significant effect on egg weight with larger females laying larger eggs ($F_{1, 115}=8.65$, $P=0.004$). Measurements of tarsus length for experiment 1 were not available. Since female offspring with red ringed fathers are larger than those with green ringed fathers it may be predicted that they would lay larger eggs. Larger eggs have several advantages in terms of hatchability and post hatching chick survival as previously discussed.

It has been well documented that developing female zebra finches are more vulnerable to nutritional stress than males. Female mortality is higher under conditions of low diet quality or enlarged brood sizes at the embryonic stage (Rutstein *et al.* 2004a), during the nestling phase (Bradbury and Blakey 1998; Kilner 1998; Birkhead *et al.* 1999) and after fledging (De Kogel 1997). Martins (2004) found that females, but not males, reared on a low quality diet had slower growth rates and lower body mass at fledging and suggested that this might account for the increased post hatching mortality of female chicks on poor diets.

Since the differences in tarsus length are related to the father's ring colour rather than foster father's ring colour it is likely that they are due to variations in egg composition rather than nestling provisioning. As increased mortality of females under poor dietary conditions has been found at the embryonic stage (Rutstein *et al.* 2004a) this further supports the suggestion that subtle changes in egg composition may affect females differently from males. Increased levels of yolk testosterone may be responsible for the increase in tarsus length found here. Testosterone has been shown to affect growth in both canaries (Schwabl 1996b) and black-headed gulls (Eising *et al.* 2001) measured during the nestling phase. We found no difference in growth rate at this stage but this does not eliminate the possibility of a more subtle effect on growth that only becomes evident once adult size is attained, particularly as females appear to be more susceptible to alterations in egg composition.

Female Offspring Reproductive Characteristics

Father's ring colour had no effect on offspring clutch size but female offspring with red ringed fathers laid larger eggs. Increased egg size has positive consequences for chick survival. This demonstrates 'grand maternal effects' where the benefits of maternal effects from one generation are passed on to the subsequent generation. Offspring egg size is related to both offspring mass (Fig. 3c) and offspring tarsus length (Fig. 3d). As tarsus length of female offspring is affected by father's attractiveness (Fig. 1a and 1b) this is the most likely the mechanism whereby egg size is increased.

Male Offspring Attractiveness

There was a non-significant trend for the sons of red ringed fathers to be more attractive themselves (Fig. 4). As father's attractiveness was artificially and randomly assigned using coloured leg rings then this result is not due to genetic effects and must relate to egg composition. Male attractiveness was assessed as the result of female choice; we did not measure individual components of attractiveness to examine how they differed between males. Male sexual attractiveness in zebra finches is a multi component signal; females choose males on the basis of song rate, beak colour and behaviour (Collins and tenCate 1996). Yolk testosterone may affect any of these traits and further work is needed to examine this.

In an experimental study on zebra finches, yolk testosterone levels were elevated through injection with testosterone. In mate choice trials, both male and female offspring from experimental eggs were preferred over controls (von Engelhardt 2004). Strasser and Schwabl (2004) found that male house sparrows hatching from testosterone treated eggs had

significantly larger patches of black throat feathers, known as 'badges', than control males. Badge size in male house sparrows is correlated with their attractiveness to females (Veiga 1993), although this was not measured.

3. Additional Results

Final Size

Weight

There was a trend in experiment 1 for female offspring to be heavier than male offspring (Fig. 2a). As this result is not related to male ring colour it is unlikely to be due to egg composition and the greater sensitivity of females to nutritional conditions during growth, as was suggested by the results for final tarsus. This difference may simply reflect the slight differences between the sexes in mass that have been recorded for zebra finches in the wild (Zann 1996).

Final weight decreased with hatching order in experiment 2 (Fig. 2b) and when both experiments were analysed together (Fig. 2c). This is expected as later hatched chicks tend to attain lower mass and tarsus measurements. It is confirmed by the finding that final tarsus length decreases with hatching order for experiment 2 (Fig. 1c) and when both experiments are analysed together (Fig. 1e).

Rate of Maturity

We found no effect of male ring colour on the rate of maturity. This is in contrast to a study on black-headed gulls (*Larus ridibundus*) where testosterone treatment was found to increase the rate at which birds developed their adult plumage (Eising *et al.*, submitted).

Dominance

Unlike the studies of Schwabl (1993) and Strasser and Schwabl (2004) we did not find any relationship between father's ring colour and dominance as adults. In this study it was intended to assess dominance on the basis of objective measurements of the amount of time spent defending the food source and the number of aggressive encounters. This would have allowed a dominance 'score' to be obtained. However, due to the lack of consistency in the birds' behaviour, a more subjective method using behavioural observations to assess dominance was also used. The resulting data was binomial and contained no information as to the level of dominance displayed by each bird. An alternative design to this experiment,

which would allow dominance to be measured rather than assigned, might have been more useful. However, although zebra finches fight over resources there appears to be little evidence of the existence of dominance hierarchies either in the wild or in captive colonies (Zann 1996). Dominance appears to be a relatively fluid behaviour dependant on individual circumstances with relatively few birds maintaining a consistent status.

Summary

The objective of the experiments presented in this and the previous chapter was to examine the effects of yolk testosterone on offspring development in the zebra finch. We compared the development of chicks hatching from eggs laid for red ringed and green ringed males as previous work has shown that eggs laid for red ringed males contain higher levels of yolk testosterone (Gil *et al.* 1999). Although we did not find in our experiments that eggs laid for red ringed males contained higher levels of yolk testosterone, we found evidence for maternal effects that are comparable with previous studies on the effects of elevated yolk testosterone.

Our study was completed in two experimental replicates. Interestingly there is more evidence of maternal effects in the first replicate, undertaken in the springtime, than in the second replicate which was carried out in the autumn. In the first experiment females laying eggs for red ringed males showed a greater increase in egg size with laying order, chicks hatching from these eggs begged more and showed greater than expected tarsus growth in relation to hatching order. Female offspring with red ringed fathers were larger and laid larger eggs. Male offspring with red ringed fathers tended to be more attractive themselves. These results suggest increased investment in egg composition by females mated to red ringed males.

In experiment 2 there was also evidence of maternal effects relating to egg composition and mate attractiveness. As in experiment 1, female offspring with red ringed fathers were larger as adults and laid larger eggs. Male offspring of red ringed fathers tended to be more attractive.

Tarsus growth, final tarsus length and final weight in experiment 1 were not influenced by either laying or hatching order. This suggests that later hatched chicks were not at a disadvantage. Females may have increased their investment in later laid eggs that counteracts the effects of hatching asynchrony, potentially increasing reproductive success. In experiment 2, these traits were all affected by hatching or laying order.

Why such differences should exist between experimental replicates is unclear. Pilz *et al.* (2003) and Schwabl (1996a) found, in the European starling and house sparrow respectively, that clutches laid earlier in the breeding season had higher levels of yolk testosterone than those laid later. If the maternal effects we have found are due to increased yolk testosterone levels then this may support the fact that we found weaker effects in eggs laid later in the season.

Due to the design of our experimental study we were not able to attribute the maternal effects we found to increased levels of yolk testosterone. A more accurate method of assessing the effects of increased yolk testosterone would be through experimental manipulation of concentration by injection. A study of this type has recently been undertaken (von Engelhardt 2004). Von Engelhardt found that testosterone treatment had no effect on survival to fledging, for female chicks the begging rate at hatching was increased and growth was enhanced. Finally, chicks hatching from testosterone treated eggs were more attractive as adults in choice tests. These results agree with our findings on survival and attractiveness of the offspring as adults. Although we did not find any sex specific differences in time spent begging and growth, we did find in experiment 1 that begging was increased in eggs laid for red ringed males and in both experiments that final adult size was greater for females.

Results Appendix

Final Tarsus

Experiment 1

Variable	df	F	p
Male Ring Colour	1, 13	9.67	0.0081
Foster Male Ring Colour	1, 12	3.74	0.0771
Female Mass	1, 26	0.44	0.5125
Chick Sex	1, 27	3.75	0.0632
Broodsize	1, 27	0.75	0.3950
Hatching Order	1, 25	0.49	0.4886
Male Ring Colour*Chick Sex	1, 27	16.14	0.0004

Experiment 2

Variable	df	F	p
Male Ring Colour	1, 20	5.9	0.0247
Foster Male Ring Colour	1, 18	0.47	0.5027
Female Mass	1, 29	0.23	0.6362
Chick Sex	1, 29	8.17	0.0078
Broodsize	1, 29	2.55	0.1210
Laying Order	1, 29	28.61	<0.0001
Male Ring Colour*Chick Sex	1, 29	19.92	0.0001

Experiment 1 and 2

Variable	df	F	p
Male Ring Colour	1, 37	12.1	0.0013
Foster Male Ring Colour	1, 36	3.47	0.0709
Female Mass	1, 57	0.08	0.7802
Chick Sex	1, 59	6.71	0.0120
Experiment Number	1, 59	0.59	0.4456
Laying Order	1, 59	10.54	0.0019
Male Ring Colour*Chick Sex	1, 59	11.32	0.0014

Final Weight

Experiment 1

Variable	df	F	p
Male Ring Colour	1, 13	1.37	0.2621
Foster Male Ring Colour	1, 12	0.31	0.5883
Female Mass	1, 26	0.09	0.7609
Chick Sex	1, 28	4.01	0.5510
Hatching Order	1, 26	0.11	0.7482
Broodsize	1, 26	0.05	0.8238

Experiment 2

Variable	df	F	p
Male Ring Colour	1, 20	0.26	0.6166
Foster Male Ring Colour	1, 17	0.00	0.9875
Female Mass	1, 34	0.07	0.7991
Chick Sex	1, 30	0.06	0.8062
Laying Order	1, 34	13.97	0.0007
Broodsize	1, 34	0.07	0.7919

Experiment 1 and 2

Variable	df	F	p
Male Ring Colour	1, 35	0.29	0.5918
Foster Male Ring Colour	1, 36	0.27	0.6079
Female Mass	1, 55	0.11	0.7418
Chick Sex	1, 56	0.27	0.6041
Experiment Number	1, 60	2.77	0.1014
Hatching Order	1, 62	12.06	0.0009

Rate of Maturity

Experiment 1 and 2

Variable	df	F	p
Male Ring Colour	1, 26	0.04	0.8383
Foster Male Ring Colour	1, 25	0.01	0.9434
Female Mass	1, 22	1.22	0.2807
Chick Sex	1, 23	2.67	0.1156
Experiment Number	1, 22	0.43	0.5171
Hatching Order	1, 21	0.01	0.9412

Female Offspring Clutch Size

Experiment 1 and 2

Variable	df	F	p
Male Ring Colour	1, 21	1.58	0.2221
Foster Male Ring Colour	1, 20	2.49	0.1306
Female Mass	1, 5	2.7	0.1610
Experiment Number	1, 4	3.01	0.1577
Hatching Order	1, 4	2.55	0.1856
Offspring Mass	1, 3	1.55	0.3015
Offspring Tarsus Length	1, 2	0.13	0.7537
Broodsize	1, 3	0.33	0.6040

Offspring Egg Mass

Experiment 1 and 2

Variable	df	F	p
Male Ring Colour	1, 19	8.48	0.0089
Foster Male Ring Colour	1, 18	0.45	0.5087
Offspring Mass	1, 117	15.45	0.0001
Broodsize	1, 116	0.29	0.5902
Experiment Number	1, 116	0.24	0.6243
Offspring Laying Order	1, 117	5.93	0.0164
Offspring Tarsus Length	1, 116	17.84	<0.0001

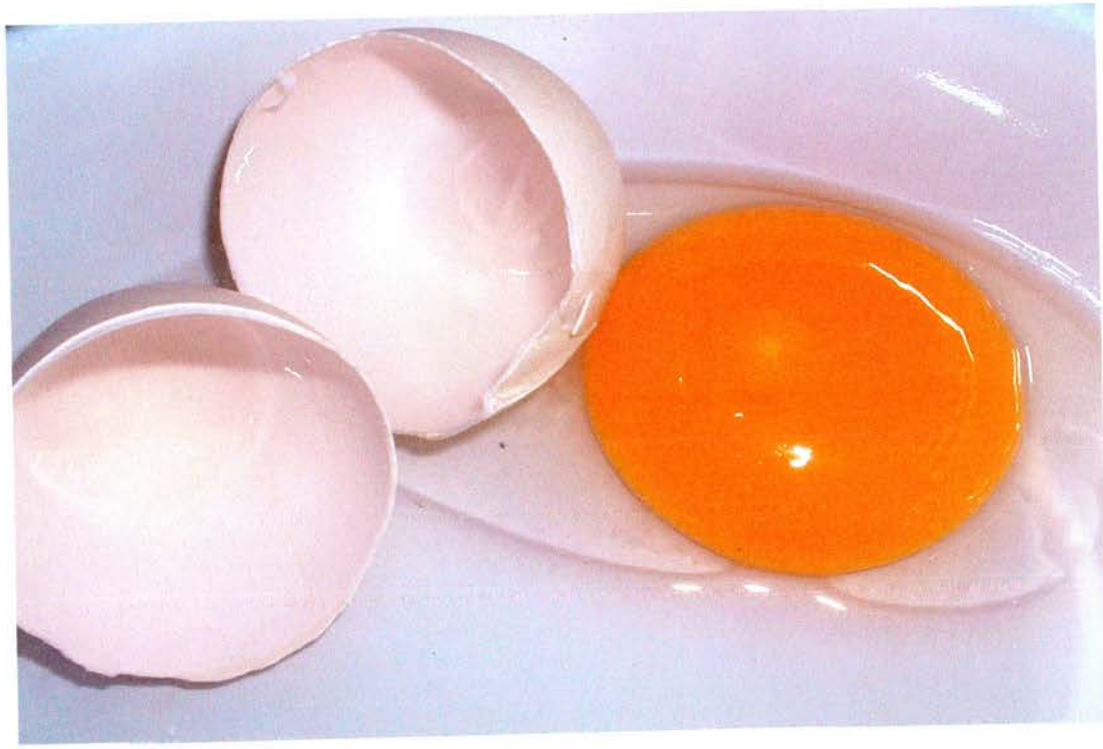
Attractiveness of Male Offspring

Experiment 1 and 2

Variable	df	F	p
Male Ring Colour	1, 12.4	4.26	0.0606
Female Mass	1, 2	2.36	0.2639
Hatching Order	1, 2	2.36	0.2639
Weight Difference	1, 2	0.02	0.9011
Tarsus Difference	1, 2	1.82	0.3100

4

Antioxidants and Mate Attractiveness



It may benefit females to make a greater investment in reproduction when mated to an attractive male (see chapter 1). In birds one of the ways that females can alter investment in reproduction is through changes in egg composition. Antioxidants are a component of egg composition. They influence many essential processes during embryonic and chick development and offer protection against substances known as free radicals.

Free Radicals

Free radicals are atoms, molecules or compounds capable of independent existence (hence the term 'free') that have one or more unpaired electrons (Halliwell and Gutteridge 1999). The possession of unpaired electrons makes free radicals unstable and reactive, although this reactivity varies over a broad spectrum. Free radicals cause damage to all types of biomolecules including DNA, proteins, carbohydrates and lipids resulting in tissue injury, disease and cell death.

A wide variety of free radicals exist in biology and are produced as part of normal body processes such as metabolism, phagocytosis, certain enzyme reactions, and exercise. Free radical production may be increased by external factors such as pollutants, ultra-violet light, toxins and radiation. The most abundant free radical produced within the body is the superoxide radical created during cellular respiration within the mitochondria.

The effect of free radicals has been extensively studied but only on human health. It is proposed that they play a central role in ageing and in the cause of several diseases such as cataracts, cancers, arthritis and heart disease. For other illnesses free radicals are a consequence of the disease process that may then result in further damage. It is claimed that up to 70% of chronic diseases are preventable through eating a healthy diet rich in antioxidants and avoiding pollutants and toxins (Lachance *et al.* 2001).

The Antioxidant Network

As free radicals are continuously produced within living tissues, defences, in the form of antioxidants, have developed to protect against them. Antioxidants are a group of substances produced by the body or gained through the diet that can prevent the formation of free radicals or remove them. Within the body there is an integrated antioxidant network consisting of three key levels of defence. At the first level are antioxidant enzymes that prevent the formation of free radicals by removing precursors or by inactivating catalysts. This process is inefficient and some free radicals are inevitably produced. The second stage involves agents that remove or 'scavenge' free radicals. These include the more familiar

antioxidants such as vitamin E, carotenoids and vitamin C. Finally, at the third stage of defence there are enzymes, which repair or eliminate the damage caused by any remaining free radicals.

Antioxidants are found throughout animal tissues in cell organelles, sub-cellular compartments and in the intracellular spaces. Antioxidants within the network work in association with each other to form an integrated system. This cooperation between antioxidants is important in order to give the maximum protection. For example, when vitamin E neutralizes a free radical it can be converted back into an antioxidant by vitamin C, which donates electrons to vitamin E bringing it back to its antioxidant state. There is no 'best' antioxidant and each has its own unique role in defence depending on the tissue under attack and the particular free radical responsible for the attack. Different antioxidants are found in different tissues where they offer protection against the particular free radicals produced by those tissues (Packer and Coleman 1999).

Uses of Free Radicals

Antioxidant defences seem to control the production of free radicals rather than eliminate them completely. Total removal of free radicals may be energetically costly to achieve and it appears there are many examples where free radicals are used in living systems for a useful purpose. Low levels of free radicals have been shown to stimulate cell division and differentiation and may have a role in cell communication (Halliwell and Gutteridge 1999). The cells that line the interior of blood vessels make the free radical nitrogen oxide. Some nitrogen oxide diffuses into the underlying smooth muscle where it causes the muscle to relax and subsequently dilate the blood vessels and reduce blood pressure. Phagocytic cells form part of the innate immune system. Their role is to detect foreign particles such as bacteria, engulf and kill them. Phagocytic cells kill invading microbes by bombarding them with free radicals (Halliwell and Gutteridge 1999; Surai 2002).

Some enzymes use free radicals to assist them in their catalytic reactions. Bombardier beetles (*Brachinus fumans*) spray their attackers with hot fluid containing quinones. The beetles have sacs containing hydroquinones and the free radical hydrogen peroxide (H_2O_2). The hydroquinone is explosively oxidized by a peroxidase enzyme using H_2O_2 to assist in the reaction producing p-Benzoquinone (Halliwell and Gutteridge 1999). The reaction is accompanied by a temperature rise of up to 100°C and a build up of pressure. The insect then uses the hot spray to deter predators.

Oxidative Stress

When the production of free radicals exceeds the abilities of the antioxidant defences to remove them then the antioxidant / free radical balance is upset. This imbalance is known as oxidative stress and it is under these circumstances that cell damage can occur. Cells can usually tolerate mild oxidative stress by synthesising extra antioxidants in order to restore the balance. However, these mechanisms are limited and once free radical production exceeds the ability of the antioxidant system to neutralise them then damage can occur.

Antioxidants in egg yolk

Antioxidants provide free radical protection to the egg contents and embryo during development and to the chick on hatching. Two groups of antioxidants are found within eggs; water-soluble antioxidants found in the albumen, and lipid soluble ones found in the yolk. The lipid soluble yolk antioxidants vitamin E, vitamin A and carotenoids are particularly important.

Carotenoids

Carotenoids are biologically active pigments. They are the most numerous and widespread group of pigments in nature (Surai 2002). Over 600 are known to exist producing colours ranging from pale yellow to dark red and they are responsible for the colour of egg yolk. Carotenoids are synthesised by higher plants, algae and some microorganisms: animals must get them from their diet. In plants they play an important role in protecting the plant tissues from free radicals formed during photosynthesis. Carotenoids are found in the lipid portions of animal tissues including cell membranes and cytoplasmic lipid droplets. Some carotenoids e.g. alpha and beta-carotene, are precursors for vitamin A (Halliwell and Gutteridge 1999; Packer and Coleman 1999).

The main storage organ for carotenoids is the liver although they are also found distributed throughout the body in other organs including the heart, muscles, spleen, body fat and blood plasma. Carotenoids can also be stored in the integument and are responsible for the colouration of feathers and other structures in some species e.g. beak and legs. As carotenoids are not only antioxidants and colourants but also act as immunostimulants they have attracted interest as sexual signals (von Schantz *et al.* 1999; Olson and Owens 1998). Healthy birds in good condition need fewer carotenoids for immune function and so can devote more to ornamental display. In this way colouration can act as an 'honest signal' of mate quality. This hypothesis has been tested for several species (blue tits (*Parus caeruleus*) –

Senar *et al.* 2003; blackbirds (*Turdus merula*) - Faivre *et al.* 2003; greenfinches (*Carduelis chloris*) – Saks *et al.* 2003; zebra finches - Blount *et al.* 2003; McGraw and Ardia 2003). Beak colouration in zebra finches is known to be carotenoid based. Blount *et al.* (2003) showed that males given carotenoids as a dietary supplement were more immunocompetent and more attractive to females than controls.

Vitamin E

Vitamin E is the generic term given to a group of substances that have similar biological activity and chemical structure. The group consists of alpha, beta, gamma and delta tocopherol and tocotrienol. Tocopherols and tocotrienols are pale yellow oils produced by higher plants. Tocopherols are found in the green parts of plants whereas tocotrienols are found in bran and germ parts of the seeds (Halliwell and Gutteridge 1999). The antioxidant abilities of tocotrienols and tocopherols vary with alpha-tocopherol and tocotrienol being the most important and delta-tocopherol and tocotrienol the least important. Alpha tocopherol is primarily located in cell membranes where it plays an important role in stabilising the membrane and protecting membrane lipids from peroxidation (Surai 2002).

Vitamin A

Vitamin A or retinol does not come from the diet. It is synthesised via enzyme activity in the gut wall from certain carotenoids. Over 50 carotenoids can generate vitamin A, although the most important is probably β-carotene (Packer and Coleman 1999). Vitamin A is essential for cell growth and differentiation, is important in activating genes and in vision. Deficiency of vitamin A results in growth retardation and bone abnormalities.

Importance of Antioxidants

Antioxidants play an important role in embryonic growth, hatching success and the development and survival of the chick. Carotenoids are important in the promotion of cell differentiation and the regulation of cell proliferation, both of which are vital for embryonic growth (Surai *et al.*1999; Halliwell and Gutteridge 1999), and they function best as antioxidants at low oxygen tensions such as those found in the developing embryo. Eggs with reduced vitamin E content show reduced fertility, slowed embryonic growth, poor hatchability and reduced viability of the newly hatched chicks (Surai 2002).

Egg yolk contains high levels of lipids, particularly polyunsaturated fatty acids (PUFAs). These are important constituents for biological membranes, are precursors of substances such as hormones and provide 90% of the energy needed by the growing embryo. Free radicals can break down PUFAs through peroxidation. Antioxidants prevent this, allowing lipids to be used during embryonic growth or stored for use following hatching.

During development lipids and lipid soluble antioxidants (Vitamin A, Vitamin E and carotenoids) are absorbed into the embryo where they accumulate throughout the body tissues although the liver receives the most. At hatching the liver contains high levels of both PUFAs and lipid soluble antioxidants. This lipid store is essential for post hatching development and the accompanying antioxidants protect it against the oxidative stress that occurs at hatching. As the embryo develops its metabolic rate, and therefore its oxygen consumption, increase thereby increasing the risk of lipid peroxidation. This increase is greatest after the egg has been pipped and on hatching with the onset of pulmonary respiration when the lungs begin to function (Tazawa and Whittow 2000).

At hatching the immune system, digestive system, nervous system and endocrine system of the chick are immature. They actively develop during the first week of life, a process that uses the lipid and antioxidant store within the liver and those in the residual yolk. These stores are also the initial source of nutrients, as the ability of the digestive system to absorb lipids and antioxidants is not well developed at this stage. The main immunological protection at hatching comes from maternally derived immunoglobulins deposited in the yolk and absorbed into the embryo. Antioxidants protect this passively acquired immunity against free radical damage. Chicks hatching from eggs with higher concentrations of carotenoids and vitamin E show an increased immune response (Blount, unpublished data, Haq *et al.* 1996). Adequate antioxidant protection of the chick at hatching is therefore crucial to its future survival. There is a direct relationship between the level of vitamin E in the newly hatched chick's liver and its viability (Surai 2002).

Antioxidants are a valuable resource for successful chick development and survival. However, the laying female also has an antioxidant requirement. This conflict results in a trade-off for the female between provisioning eggs and maintaining her own health and body condition. For carotenoids and vitamin E this trade-off is particularly relevant as these antioxidants can only be gained from the diet suggesting that they may be limiting (Blount *et al.* 2002; Royle *et al.* 1999).

Antioxidants and Mate Attractiveness

Differential allocation theory (Burley 1988b) predicts that females should invest more in a reproductive attempt when mated to an attractive male (see chapter 1). As antioxidants are an essential but limited resource, females may compromise their own health by depositing higher concentrations in the eggs that they lay for attractive males. This potentially increases the survival chances of their offspring and their own lifetime reproductive fitness but at a cost to future survival.

Aims

I investigated the relationship between yolk antioxidants and mate attractiveness in the zebra finch (*Taeniopygia guttata*). I examined the deposition of carotenoids, vitamin A and vitamin E in eggs laid for attractive and unattractive males using High Performance Liquid Chromatography (HPLC). Mate attractiveness can be manipulated in zebra finches using coloured leg rings. Studies have shown that female zebra finches find males wearing red leg ring attractive whereas green rings are unattractive (Burley *et al.* 1982; see Chapter 1).

Carotenoids were measured as total carotenoids present in yolk; I did not distinguish between individual carotenoids. Vitamin E was measured as the amount of alpha-tocopherol and gamma-tocopherol present. Alpha-tocopherol is the most important constituent of vitamin E in terms of antioxidant potential; gamma-tocopherol is abundant in the yolk of zebra finch eggs as a reflection of the level found in the commercially available diet (Surai, unpublished data).

Results are given as the concentration of antioxidants in yolk and the total amount in whole yolk. It was not possible to compare the overall investment for the entire clutch with mate attractiveness since females laid clutches of different sizes. It was predicted that eggs laid for red ringed males would have higher levels of vitamin E, vitamin A and carotenoids.

Methods

General Methods

Where possible females with no previous breeding experience were used (8 out of 10) thereby eliminating any potential carry over effects of male attractiveness from an earlier breeding attempt (Rutstein *et al.* 2004b; Rutstein *et al.* 2005). The two remaining females had only bred once, 6 months previously, with males whose attractiveness had not been manipulated. Females were weighed, then tarsus measurements and fat scores were recorded. Females were

paired with either a red or green ringed male and housed in individual breeding cages. Nest boxes were checked twice daily. Eggs were collected on the day they were laid, weighed, measured, numbered with a non-toxic pen and stored at -70°C awaiting yolk analysis. Eggs removed from nests were replaced with dummy eggs thereby allowing the natural clutch size to be maintained.

Antioxidant Extraction and Analysis

Antioxidant Extraction

A sample of yolk (100mg) was mixed with 0.7ml 5% NaCl solution and 1ml ethanol, 2ml hexane was added and the mixture was homogenized for 30 seconds. Samples were then centrifuged and the hexane phase containing vitamins A, E and carotenoids, was collected. Extraction with hexane was repeated and the combined collection was dried under a stream of N_2 gas. The resulting residue was dissolved in methanol / dichloromethane (1:1 v/v) and centrifuged ready for HPLC analysis.

Carotenoid concentrations were determined using a Spherisorb type S30DS2, 5μ C18 reverse-phase column, 25cm x 4.6mm (Phase Separation, Clwyd, UK) with a mobile phase of methanol / water (97:3 v/v) at a flow rate of 1.50 ml / min. Total carotenoids were detected as a single peak at 445nm.

For vitamins A and E chromatography was performed using a Spherisorb type S3ODS2 3μ C18 reverse-phase column and mobile phase of methanol / water (97:3 v/v) at a flow rate of 1.05 ml / min. Fluorescence detection of vitamin A involved excitation and emission wavelengths of 325 and 295nm. Vitamin E detection used excitation at 295nm and emission at 330nm. Peaks were identified from standards.

Antioxidant Analysis

Yolk antioxidant concentrations were determined using high-performance liquid chromatography (HPLC). The use of HPLC as an analytical technique is expensive, therefore many of the studies undertaken using HPLC have small sample sizes where relatively few individuals are used to represent each experimental condition. Due to the high cost it was initially intended that the analysis of yolk samples would take place at St. Andrews under the guidance of Dr. Graham Kemp who has the use of an HPLC set up in Biomolecular Sciences. However, several problems occurred due to the age and inadequacy of the equipment. After a period of some 6 months spent refining the extraction technique, calibrating the procedure and

overcoming a range of problems it was decided to abandon the analysis at St. Andrews, repeat the egg collection phase of the experiment and run the analysis in the lab of Prof. Peter Surai at the Scottish Agricultural College in Ayr which is devoted to the study of antioxidants.

St. Andrews

Standard Curves

HPLC is used to identify and quantify chemical compounds. The concentration of a particular compound within a sample is determined by the amount of light of a given wavelength absorbed by that compound. To quantify antioxidants within the extracts it is necessary to construct standard curves using known standards. A number of trial extracts were run to give an indication of the levels of absorbance expected. Standards for alpha-tocopherol and retinol were bought from Sigma (Sigma-Aldrich, U.K.). Standards were diluted such that injecting a volume of 50µl gave an absorbance that matched the average of the test samples. Standard curves were then determined by injecting increasing volumes of standard starting at 10µl and increasing by units of 10µl to a maximum of 150µl for alpha-tocopherol and 100µl for retinol.

The HPLC system at St. Andrews uses manual injection of samples. Each volume injected was repeated 3 times. By using 3 repetitions for each point on the standard curve it is possible to check the repeatability of the results by calculating the coefficient of variation. When taking repeated measurements from a single sample there will be some variation in the results. The coefficient of variation gives an indication of how reliable these measurements are, and subsequently how well the equipment is working. A coefficient of variation under 5% is regarded as sufficiently reliable (P.Surai, pers. comm.).

The standard curve for alpha-tocopherol at a concentration of 7.05ppm is shown in fig.1. Retinol standard curve at a concentration of 0.377ppm is shown in fig. 2. No units are given on the Y-axis as measurement of light absorbance uses arbitrary units. The relationship between absorbance and volume of standard can be quantified by giving the coefficient of determination (r^2). This value measures how well the variability in the dependant variable (absorbance) is accounted for by the independent variable (volume of standard injected). For alpha-tocopherol the r^2 value is 0.9923 (Fig. 1), and for retinol it is 0.998 (Fig. 2). These values are extremely high which would be expected since standards are pure solutions.

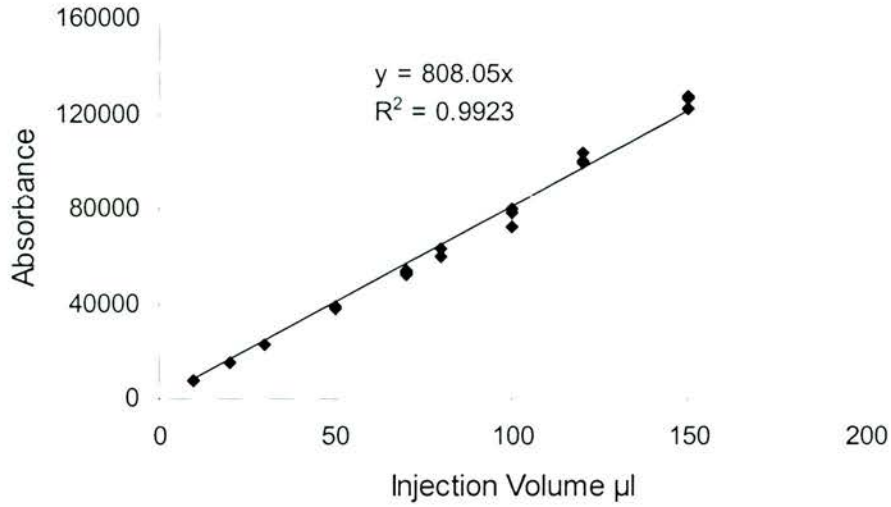


Figure 1. Standard curve for alpha-tocopherol at a concentration of 7.05ppm.

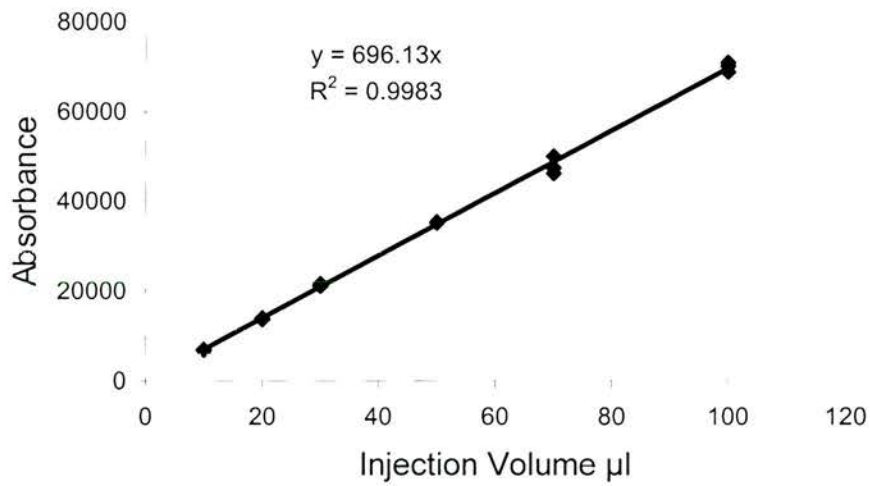


Figure 2. Standard curve for retinol at a concentration of 0.377ppm.

Since carotenoids were measured as the concentration of total carotenoids and not as individual carotenoids, no standard was used to assess concentration. Using serial dilution it was possible to show that absorption was related to the concentration of carotenoid within the sample. Fig. 3 shows the relationship between absorbance and the quantity of carotenoid. Carotenoids were measured as the amount of yolk present in the volume injected. This shows that there is a relationship between total carotenoid content within the samples and absorbance. The r^2 value is 0.85. This is lower than the value for the alpha-tocopherol and retinol standard curves which would be expected as the extract used to determine the

relationship is not a pure solution of carotenoids but contains other chemical compounds that will affect the absorbance.

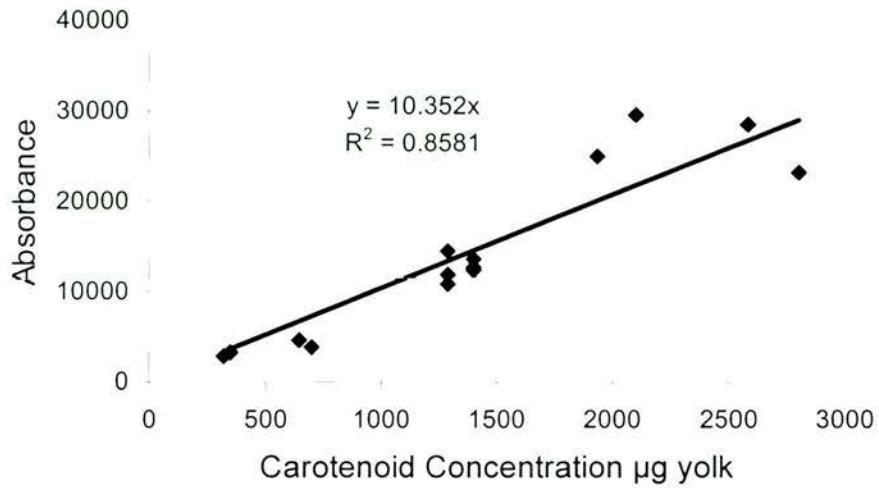


Figure 3. Standard curve for carotenoids.

Initial Results

Following extraction, samples were injected onto the HPLC column to measure the concentration of antioxidants present. Each sample was injected 3 times to determine repeatability. The volume of extract injected was adjusted if it was found to fall outwith the standard curve.

Extractions were processed in batches and stored at –70°C until HPLC analysis within 1 to 3 days. To begin with extractions were processed in small batches of 8 to 10 samples. It became apparent once two batches had been run that there were differences between the batches in terms of the concentration of antioxidants present. This was not due to batches containing samples from only one experimental group as care was taken to use equal numbers of samples from the two experimental groups within each batch. Throughout this time problems began to develop with the HPLC equipment due to its age and lack of use. There were problems with the solvent pumps, injection port and filters (frits), but the greatest problem was with the pressure across the column. The pressure across an HPLC column must remain steady for the results to be both reliable and repeatable. Pressure can fluctuate for several reasons and it was found to be rarely stable on this machine. Temperature rises within the room where an HPLC set up is working can cause retention times to drift which also leads to unreliable results. To prevent this it is necessary to work in an air-conditioned room or to

use a column oven that controls temperature fluctuations across the column. I had access to neither of these and found that retention times continually fluctuated.

Due to the problems with the HPLC set up and the apparent differences between batches it was considered sensible to process as many samples as possible and run them together. This would allow a more accurate comparison to be made as it was assumed that the problems with the HPLC equipment were causing the results to fluctuate between batches. Following this decision twenty extractions were processed in a single day. When these samples were analysed using HPLC it became apparent that the concentration of antioxidants within the samples was much less than before. I came to the conclusion that the extracts must be deteriorating during the extraction procedure. Antioxidants deteriorate under conditions of heat, light and oxygen. They were exposed to increased amounts of all of these as the extraction time increased due to processing larger batches.

As a result of immense problems with the HPLC equipment and the realisation that the entire breeding experiment would need to be repeated, I decided to abandon the analysis at St. Andrews. Peter Surai kindly allowed me to carry out the analysis in his lab at Ayr using his HPLC set-up which is calibrated for antioxidants, is situated in an air conditioned room and has an auto sampler. Repeatability with this machine does not normally vary from a coefficient of variation of 2%. Analysis at Ayr used the extraction technique and equipment column specification already described.

Statistical Analysis

To assess possible differences in yolk antioxidants with male ring colour and laying order, mixed general linear models were used with female ring number as the random factor. Male ring colour, laying order, tarsus length and female fat score were entered as the main variables. Tarsus measurements give a measure of overall body size whereas fat score indicates body condition. As weight represents a combined estimate of both body size and condition it was decided to use fat score and tarsus in the statistical analysis thereby avoiding the use of conflicting explanatory variables. Following a forward stepwise procedure tarsus length was statistically eliminated from all models at an early stage. Full tables of results are given in an appendix at the end of the chapter.

Analysis showed a significant interaction between male ring colour and laying order for carotenoids and alpha-tocopherol. Therefore an analysis of total investment in the clutch

as a whole was done using clutch size as an explanatory variable thereby allowing differences in clutch size to be controlled for.

Analysis used SAS version 8e (SAS Institute, 1998). Data were tested for normality using Kolmogorov-Smirnov tests on Minitab version 12 and transformed where necessary. Models were checked for 'goodness of fit' using residual plots (Grafen and Hails 2002).

Results

Carotenoids

Yolk Concentration

Carotenoid concentration decreased with laying order. Male ring colour had no effect on its own but showed a significant interaction with laying order ($F_{1, 36}$= 6.79, P=0.0132) (Fig. 1a). Females mated to red ringed males deposited fewer carotenoids in earlier laid eggs but more in the last laid egg compared to females mated to green ringed males. Sample size was 48 eggs.

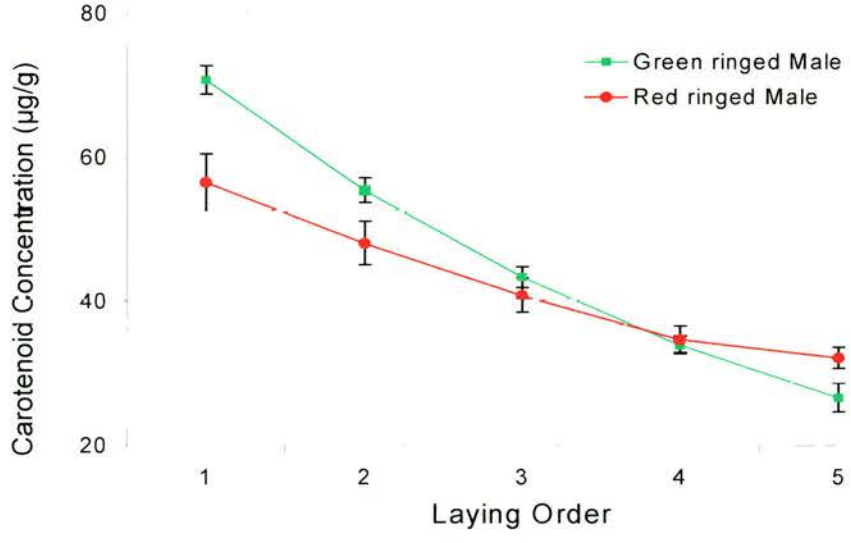


Figure1a. Fitted mean concentration of carotenoids (µg/g yolk ± s.e.) with male ring colour.

Female fat score had a significant effect on carotenoid concentration ($F_{1, 36}$= 7.41, P=0.0099) (Fig. 1b).

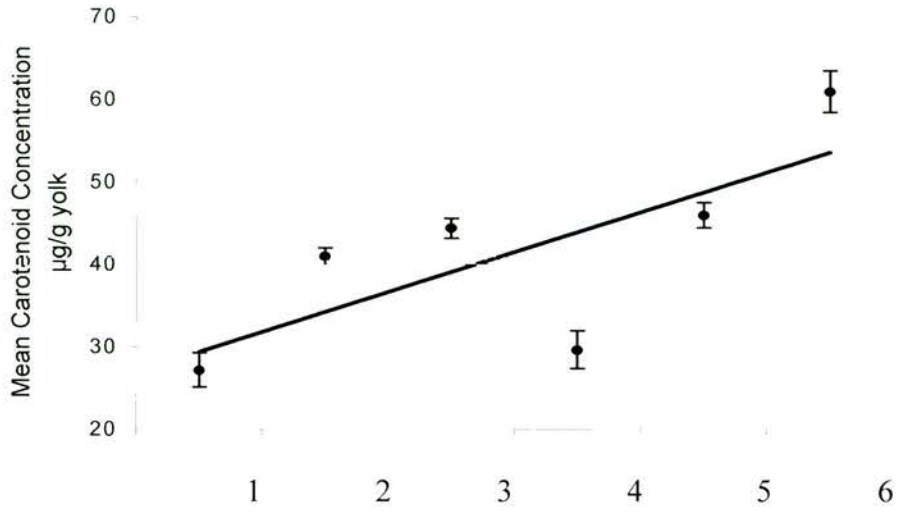


Figure 1b Fitted mean carotenoid concentration (µg/g ± s.e.) with female fat score.

Total in Whole Yolk

For total amount of carotenoids in whole yolk the interaction between male ring colour and laying order was at significance ($F_{1, 36}$ =4.08, P=0.0509) (Figure 1c). Female fat score was significantly related to mean carotenoid concentration ($F_{1, 36}$=8.15, P=0.0071) (Fig. 1d). Sample size was 48 eggs.

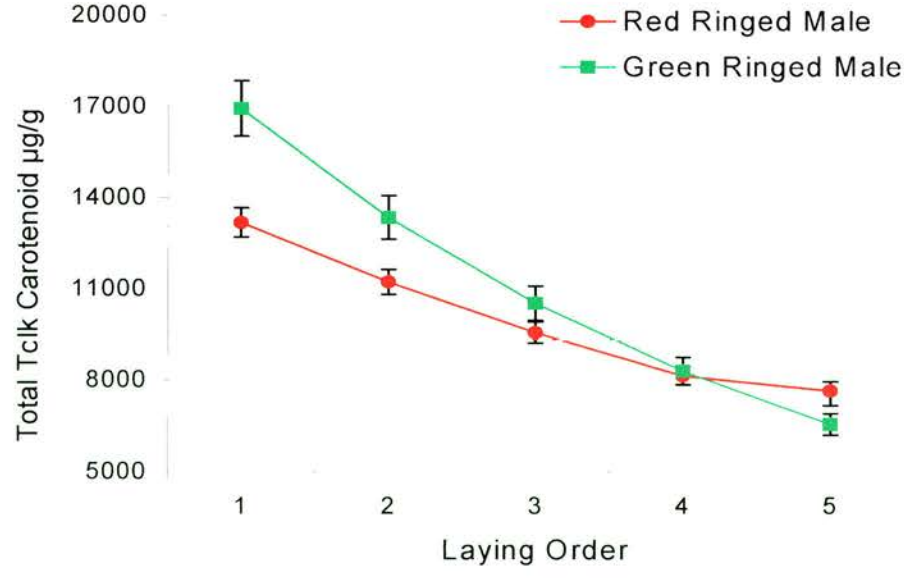


Figure 1c Fitted mean total carotenoids in whole yolk (µg ± s.e.) with male ring colour.

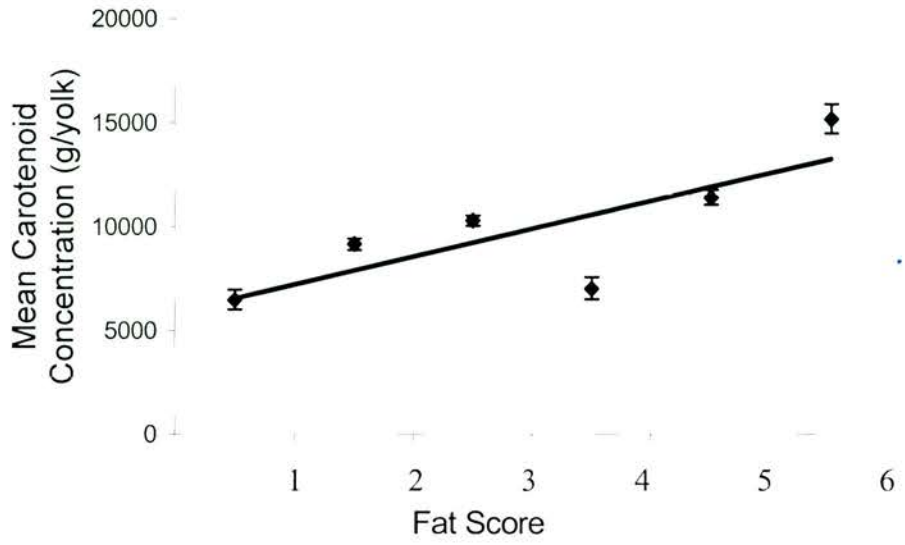


Figure 1d Fitted mean carotenoids (μg ± s.e.) in whole yolk with female fat score.

Retinol

None of the variables examined showed any relationship to retinol concentration or the total amount of retinol in yolk.

Gamma-tocopherol

Concentration

There was a significant effect of laying order ($F_{1, 37}$= 6.27, P= 0.0168)(Fig. 2a) on gamma-tocopherol concentration. Concentration declined with laying order.

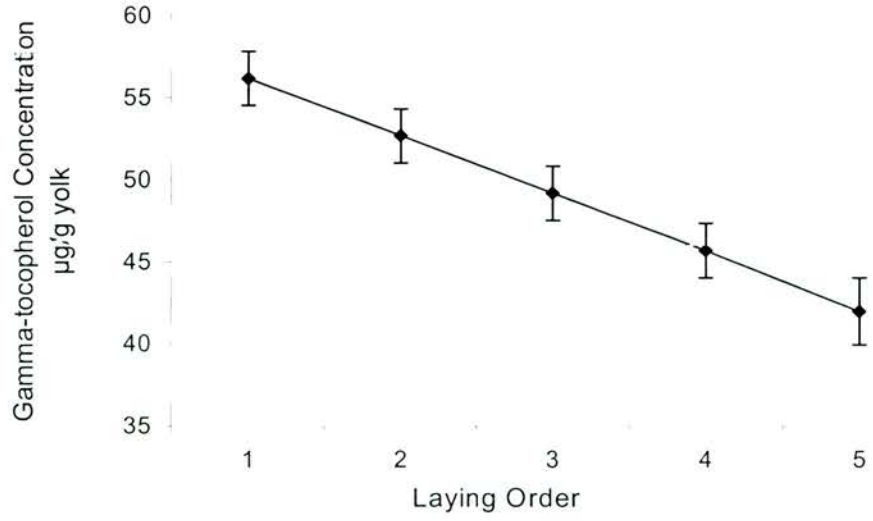


Figure 2a. Fitted mean concentration of gamma-tocopherol (μg/g yolk ± s.e.).

Total Amount in Yolk

Total amount of gamma-tocopherol in whole yolk also showed a significant decline with laying order ($F_{1, 37}$= 4.22, P=0.0471)(Fig. 2b).

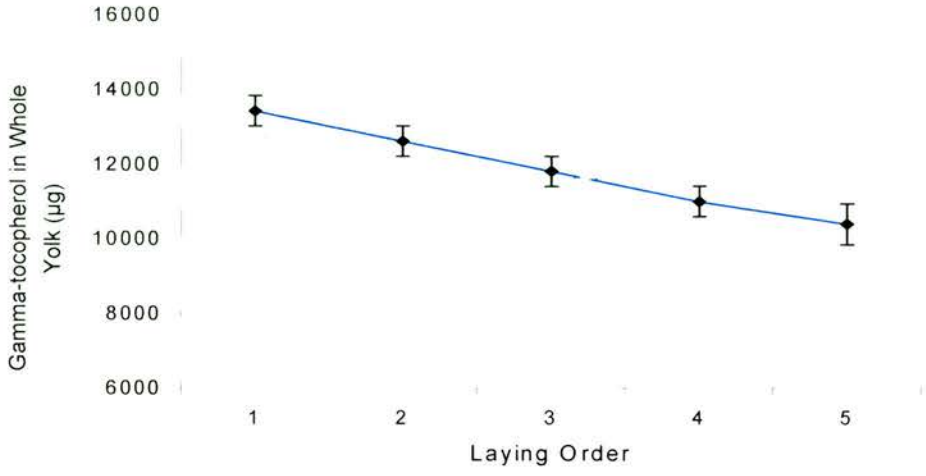


Figure 2a. Fitted mean gamma-tocopherol in whole yolk (µg ± s.e.).

Alpha-tocopherol

Concentration

Alpha-tocopherol decreased with laying order. A significant interaction between male ring colour and laying order showed that alpha-tocopherol was lower in first laid eggs for females mated to red ringed males but was greater in later laid eggs compared to that for females mated to green ringed males ($F_{1, 36}$=5.41, P=0.0259) (Fig. 3a).

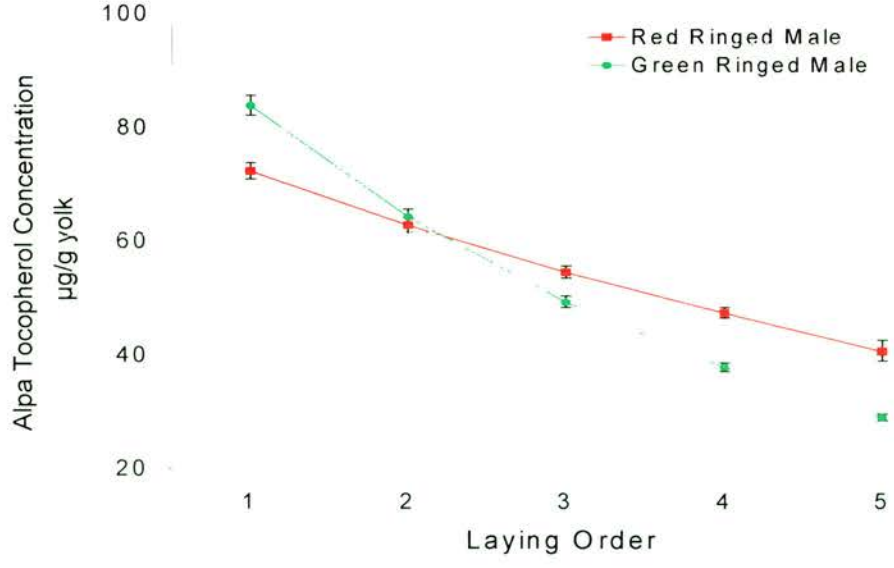


Figure 3a. Fitted mean concentration of alpha-tocopherol (µg ± s.e.) with male ring colour.

Total in Whole Yolk

There was a significant interaction between laying order and male ring colour for the total amount of alpha-tocopherol in yolk ($F_{1, 36}$= 4.36, P= 0.044)(Fig. 3b).

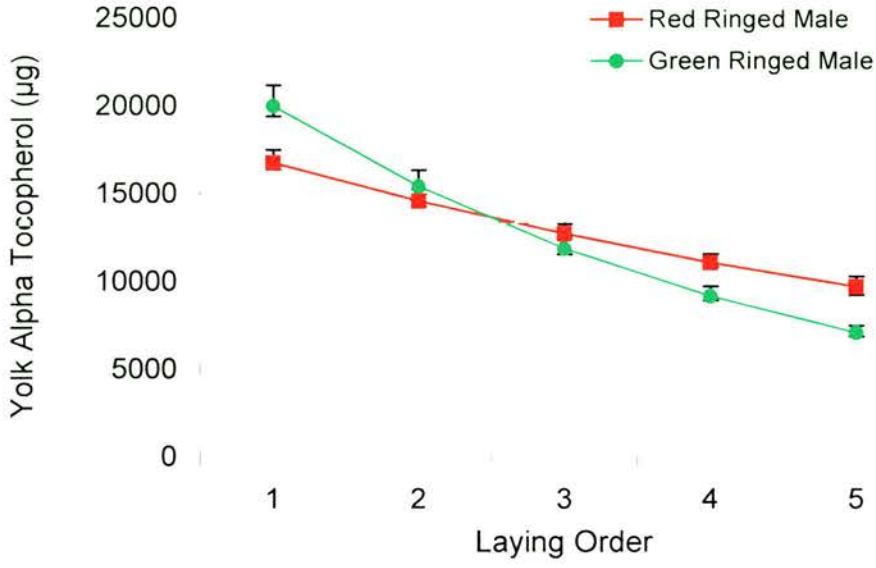


Figure 3b. Fitted mean amount of alpha-tocopherol in yolk (µg ± s.e.) with male ring colour.

Egg Weight

Egg weight was not influenced by laying order ($F_{1, 37}$=0.05, P=0.816), female fat score ($F_{1, 38}$=0.88, P=0.3537) or male ring colour ($F_{1, 8}$=1.17, P=0.3115).

Proportion of Yolk

The proportion of yolk within eggs was not influenced by laying order ($F_{1, 37}$ =0.32, P=0.5765), female fat score ($F_{1, 38}$=0.39, P=0.5341) or male ring colour ($F_{1, 8}$=0.51, P=0.4949).

Total Investment of Carotenoids and Alpha-tocopherol in Clutch

Analysis of the overall quantity of carotenoid for all eggs in a clutch together, showed that investment in the clutch as a whole did not differ between those laid for red ringed and green ringed males ($F_{1, 6}$ =4.95, P=0.0677). Overall investment of alpha-tocopherol in the clutch as a whole also showed no differences ($F_{1, 7}$ =0.67, P=0.440).

Discussion

Life history theory predicts that females should allocate resources to reproduction in a manner that increases their lifetime reproductive success (Stearns 1992; Lessells 1991). Females are expected to make a greater investment when mated to an attractive male (Burley 1988b)(see chapter 1).

Antioxidants are an important resource; they are required by the laying female to maintain her health, but they are also needed to ensure successful embryonic and post hatching development and survival of her offspring. The lipid soluble yolk antioxidants carotenoids and vitamin E are gained from the diet suggesting that they are limiting and a source of a potential conflict for the female between investment in eggs and body maintenance. I predicted that females would invest greater concentrations of antioxidants in eggs laid for red ringed males.

Although I did not find that females mated to red ringed males deposited higher concentrations of antioxidants in their eggs, the pattern of allocation for alpha-tocopherol and carotenoids with laying order differed between eggs laid for red ringed and green ringed males (Fig. 1a and 3a). This pattern was not due to differences in egg size or proportion of yolk as neither of these changed with laying order, and I found the same result for the total amount of carotenoids (Fig. 1c) and alpha-tocopherol (Fig. 3b) in whole yolks with laying order. The pattern of alpha-tocopherol concentration with laying order is particularly interesting as alpha-tocopherol has a far greater antioxidant potential than carotenoids.

I did not find that females mated to attractive males deposited more carotenoids and alpha-tocopherol in the clutch as a whole, which may suggest that females are limited in the overall amount of antioxidants which can be made available for clutch formation. However, I may have found partial support for the differential allocation hypothesis. Zebra finch eggs hatch asynchronously resulting in an age/size hierarchy within the brood (Zann 1996); later hatched chicks show a slower growth rate (Lago *et al.* 2000; Johnson *et al.* 2003) and higher mortality (Graves *et al.* 1984; Mock *et al.* 1990). By allocating antioxidants to later laid eggs females may increase the survival prospects for later hatched chicks and the chance that more chicks will survive to adulthood. Producing more chicks that will survive and reproduce themselves increases the lifetime reproductive success of the female.

Antioxidants and Laying Order

The concentration of carotenoids, gamma-tocopherol and alpha-tocopherol decreased with laying order. This has been found in most species studied to date (barn swallows (*Hirundo rustica*): Saino *et al.* 2002; lesser black-backed gulls (*Larus fuscus*): Royle *et al.* 1999; great tits (*Parus major*): Horak *et al.* 2002 and zebra finches: Royle *et al.* 2003). Dietary and supplemental feeding studies do not alter this pattern suggesting that it is a constraint of the egg laying process (Blount *et al.* 2002; Royle *et al.* 2003). In a study of yolk carotenoid levels in lesser black-backed gulls, Blount *et al.* manipulated the availability of carotenoids by supplementing the gulls with carotenoids mixed into fat and placed near the nest of the laying female. Eggs laid by supplemented females contained significantly higher levels of carotenoids but the pattern of decline with laying order was maintained.

Control of Yolk Antioxidant Levels

The synthesis and deposition of yolk within the developing follicles is controlled by oestrogen. Yolk is synthesised in the liver as vitellogenin and lipid protein complexes known as very low-density lipoproteins (VLDLs), which are then transported in the blood and deposited in the developing follicle (Johnson 2000). Lipid soluble antioxidants stored in the liver are thought to be incorporated into VLDLs, transported via the blood and subsequently deposited in the follicle. Oestrogen levels peak at about the time the first egg of a clutch is ovulated (Bahr *et al.* 1983; Sockman and Schwabl 1999) reaching a minimum with the ovulation of the last egg. This decrease in oestrogen over the laying sequence reflects the declining levels of alpha-tocopherol, gamma-tocopherol and carotenoids found in the eggs with laying order.

Oestrogen is known to have an antioxidant effect, preventing the peroxidation of lipids (Halliwell and Gutteridge 1999) and may have a role in regulating the amount of vitamin E in the liver (Feingold *et al.* 1993). Poultry administered with oestrogen showed higher plasma levels of vitamin E (Halifeoglu *et al.* 2003). Due to its antioxidant properties, higher levels of circulating oestrogen may allow the preservation of greater concentrations of both vitamin E and carotenoids within the liver at egg laying permitting higher concentrations to be transferred to the developing yolk. However, several other hormones are active during oogenesis and subtle interactions exist between them (Bahr *et al.* 1983; Johnson 2000). Hormones almost certainly influence the level of antioxidants deposited in the developing follicle, but the identity of the hormone responsible remains to be determined.

Hormones and Reproduction

Reproduction in birds must be timed to coincide with the period when resources are most abundant. Reproduction involves a complex series of events including pair formation, nest building, oviposition, incubation and nestling feeding. Males and females must synchronise their reproductive cycles to ensure success. This synchronisation depends on environmental information. Wingfield and Farner (1993) propose that two main environmental influences are involved. The first of these, 'ultimate environmental factors', bring the birds into breeding condition. Lengthening days predict the onset of spring and the optimum time to reproduce. In response to this the hypothalamus begins to produce gonadotrophin-releasing hormone (GRH). This controls the secretion of luteinising hormone and follicle stimulating hormone by the pituitary that regulated gonadal growth and function. In the male the testes develop and begin to secrete testosterone and in the female the ovaries develop and oestrogen production begins.

Once gonadal development has begun then more subtle behavioural interactions in the form of courtship displays and pair bonding become important. These are 'proximate environmental factors', which ensure that the pair can coordinate their breeding cycles and reproductive effort. Environmental factors influence female hormone levels during reproduction. Several studies have found a relationship between competition, aggression and levels of testosterone in egg yolk (Schwabl 1996a; Whittingham and Schwabl 2002; Pilz and Smith 2004). The attractiveness of the male, whether signalled by his song, courtship display or overall appearance may cause the female to alter the levels of hormones she secretes. Gwinner *et al.* (2002) found that concentrations of luteinising hormone in female European starlings (*Sturnus vulgaris*) were higher when paired with a higher quality male. Female canaries (*Serinus canaria*) exposed to the song of an attractive male deposited more testosterone in their eggs (Tanvez *et al.* 2002; Gil *et al.* 2004). Female zebra finches paired with an attractive male also deposit more testosterone in their eggs (Gil *et al.* 1999). As testosterone is a precursor of oestrogen these studies may also indicate that there were changes in the levels of oestrogen with exposure to an attractive male. These studies suggest that having an attractive mate may prompt a higher physiological reaction, which may ultimately lead to the manipulation of egg composition.

Gamma-tocopherol is not influenced by laying order or male ring colour. Gamma-tocopherol has a much lower antioxidant potential than alpha-tocopherol. In terms of which components of vitamin E are used first during times of oxidative stress, alpha-tocopherol and tocotrienol are oxidized first then beta tocopherol and tocotrienol, then gamma and finally

delta. As alpha-tocopherol is oxidised first then in the liver under influence of oestrogen it is alpha-tocopherol that is most likely to be preserved if oestrogen acts as an antioxidant. Gamma-tocopherol may be less influenced by oestrogen levels as is only oxidized once alpha and beta tocopherol and tocotrienol have been depleted.

Female Condition and Carotenoids

Female body condition measured as fat score had a significant effect on the concentration of carotenoids in yolk (Fig. 1b) and the absolute amount in whole yolk (Fig. 1d). There was no effect on vitamin A, alpha-tocopherol or gamma-tocopherol. This suggests that females in better condition, with greater fat reserves, are better able to provision eggs with carotenoids. Whereas vitamin A and E are stored mostly in the liver, carotenoids can be stored throughout the body in several areas including the integument and body fat.

Male zebra finches have been shown to prefer females that are more 'fecund' (Jones *et al.* 2001). Fecundity was defined as those females who had received an enriched diet bringing them into better condition. Supplemented females have been shown to lay larger clutches and larger eggs (Monaghan *et al.* 1996; Rutstein *et al.* 2004a) and our results suggest that they may also be able to increase egg quality, at least in terms of carotenoid deposition.

Retinol

A previous study (Royle *et al.* 2003) described an increase in retinol concentration with laying order for zebra finch eggs, although I did not find this. Royle *et al.* supplemented the birds in their study with vitamin A in their drinking water. This would increase the amount in the egg yolk, which may have helped identify any existing patterns. The amount of retinol found here was low (P. Surai, pers. comm.) making it potentially difficult to detect any patterns in deposition.

Summary

I examined the deposition of the antioxidants vitamin E, vitamin A and carotenoids in egg yolk in relation to mate attractiveness in the zebra finch. I found that females deposited alpha-tocopherol and carotenoids in different patterns with laying order depending on the attractiveness of their mate. Females paired with red ringed, attractive, males deposited more of these antioxidants in later laid eggs than females mated to unattractive, green ringed, males. Chicks hatching from later laid eggs show poorer development and survival. Therefore, by

increasing the allocation of antioxidants to these eggs the prospects for later hatched chicks may be enhanced and the lifetime reproductive fitness of the laying female increased.

Several hormones play a role in the process of egg laying and some of these are known to interact with antioxidants. Changes in the circulating hormone levels of the laying female in relation to the attractiveness of her mate may be a mechanism whereby differences in antioxidant deposition with laying order are attained.

Results Appendix

Carotenoids

Concentration

Variable	df	F	p
Male Ring Colour	1, 7	0.36	0.5700
Female Fatscore	1, 36	7.41	0.0099
Laying Order	1, 36	166.88	<0.0001
Laying Order*Male Ring Colour	1, 36	6.79	0.0132

Total in Whole Yolk

Variable	df	F	p
Male Ring Colour	1, 7	0.25	0.6358
Female Fatscore	1, 36	8.15	0.0071
Laying Order	1, 36	108.95	<0.0001
Laying Order*Male Ring Colour	1, 36	4.08	0.0509

Retinol

Concentration

Variable	df	F	p
Male Ring Colour	1, 8	0.72	0.4205
Female Fatscore	1, 38	0.30	0.5848
Laying Order	1, 37	0.04	0.8517

Total in Whole Yolk

Variable	df	F	p
Male Ring Colour	1, 8	0.29	0.6034
Female Fatscore	1, 38	0.00	0.9810
Laying Order	1, 37	0.33	0.5682

Gamma-tocopherol

Concentration

Variable	df	F	p
Male Ring Colour	1, 8	2.07	0.1881
Female Fatscore	1, 38	0.00	0.9885
Laying Order	1, 37	6.27	0.0168

Total in Whole Yolk

Variable	df	F	p
Male Ring Colour	1, 8	1.38	0.2742
Female Fatscore	1, 38	0.02	0.8856
Laying Order	1, 37	4.22	0.0471

Alpha-tocopherol

Concentration

Variable	df	F	p
Male Ring Colour	1, 8	2.03	0.1917
Female Fatscore	1, 38	0.30	0.5891
Laying Order	1, 36	57.71	<0.0001
Laying Order*Male Ring Colour	1, 36	5.41	0.0257

Total in Whole Yolk

Variable	df	F	p
Male Ring Colour	1, 8	1.87	0.2092
Female Fatscore	1, 38	0.00	0.9987
Laying Order	1, 36	46.29	<0.0001
Laying Order*Male Ring Colour	1, 36	4.36	0.0440

5

Mechanisms of Androgen Deposition in Yolk

The occurrence of steroid hormones in egg yolk has been most extensively studied in birds. In several species androgen levels have been found to vary within clutches in relation to laying order. Patterns of increasing and decreasing yolk testosterone levels have been found and it has been suggested that this variation is adaptive. Yolk androgens have been shown to have a beneficial effect on several aspects of chick development including growth, dominance and adult morphology (e.g. Schwabl 1993; Eising *et al.* 2001). Increasing levels of androgens with laying order have been proposed to ameliorate the effects of hatching asynchrony (Schwabl 1993; Eising *et al.* 2001) whereas decreasing levels facilitate brood reduction (Schwabl *et al.* 1997c).

Androgen levels also vary between clutches in relation to social and environmental factors such as photoperiod (Schwabl 1996a), nesting density (Reed and Vleck 2001; Groothuis and Schwabl 2002; Mazuc *et al.* 2003; Pilz and Smith 2004), aggression (Whittingham and Schwabl 2002), food supply (Verboven *et al.* 2003) and mate attractiveness (Gil *et al.* 1999; Gil *et al.* 2004; Tanvez *et al.* 2004). By altering yolk testosterone levels in relation to environmental conditions females may enhance offspring survival. For example, black-headed gulls (*Larus ridibundus*) nesting on the periphery of the colony lay eggs with higher testosterone levels. As the predation rate at the edge of the colony is high, increased yolk testosterone may enhance the growth and locomotor development of the chicks allowing them to escape predators more easily (Groothuis and Schwabl 2002).

What remains unclear is how females vary yolk androgens. What are the mechanisms responsible for differing concentrations of androgens within yolk? Determining the method of increased yolk androgen deposition is important in understanding adaptive variation. It is also important in appreciating the potential cost that elevated androgen levels may have to the laying female.

Steroid Production Within the Ovaries

In female vertebrates steroid hormones are produced within the ovaries. In the ovaries of birds the follicles develop together in a distinct hierarchy. The largest follicle, which will ovulate first is termed the F_1 follicle, the next largest is the F_2 follicle and so on. The number of follicles developing simultaneously is dependant on the final clutch size of the female. Each follicle consists of concentric layers of tissue surrounding the oocyte. In terms of steroid production the granulosa, theca interna and theca externa cell layers are the most important (Bahr *et al.* 1983; Johnson 2000).

Ovarian steroidogenesis has been extensively studied in domestic poultry. Pituitary gonadotrophins control the development of the follicular hierarchy and hormone production. In response to the secretion of luteinising hormone (LH) and follicle stimulating hormone (FSH), enzymes in the granulosa and theca cell layers convert cholesterol to several steroid hormones (Etches 1996).

Steroid production in these cell layers has been described in terms of a 3-cell model (Porter *et al.* 1989). The granulosa cell layer produces progesterone, which is converted to testosterone in the theca interna cell layer. Then within the theca externa layers, testosterone is aromatised to oestrogen. The enzyme activity of these 3 cell types changes during follicle maturation. Oestrogen production by the theca externa cells declines as the follicle develops, whereas progesterone production by the granulosa cells increases being highest at the F_1 stage shortly before ovulation. Testosterone production remains relatively steady until the F_1 stage when it drops rapidly (e.g. Bahr *et al.* 1983).

Support for this pattern of hormone secretion comes from studies of the concentration of hormones within yolk layers. Yolk is deposited around the oocyte in concentric spheres with the layers closest to the oocyte deposited early in follicular growth and the outermost layers added shortly before ovulation. Lipar *et al.* (1999a) analysed the hormone concentration in the yolk layers of eggs from two species: the red-winged blackbird (*Agelaius phoeniceus*) and dark-eyed junco (*Junco hyemalis*). The pattern of steroid concentration within these layers matched that predicted by the pattern of steroidogenesis found by Bahr *et al.* (1983). Progesterone concentration increased towards the outer yolk layers and oestrogen concentration was highest in the centre and declined towards the outer yolk layers.

Within Clutch Patterns of Testosterone Concentration

These shifting patterns of enzyme activity and subsequent steroid production at different stages of follicular growth may account for the differing levels of androgen concentration found within the eggs of a clutch.

Steroid hormones are fat-soluble and therefore move easily across cell membranes. As a result of this they are never stored and leave the cells where they are produced almost immediately (Nelson 2000). The growing follicles are highly vascularized and the blood flow within the ovary is greatest around the largest follicles (Johnson 2000). This allows steroid hormones to enter the circulation where they are carried bound to soluble carrier proteins. As all follicles develop simultaneously, they will be subject to steroid hormone production from the other follicles via the circulation. The smaller follicles, which are the last to ovulate and

ultimately become the later laid eggs within a clutch, will be exposed to steroid production from all the other follicles within the hierarchy and may accumulate higher concentrations of androgens. This may be the mechanism responsible for increasing testosterone concentration across laying order found in several species (e.g. Schwabl 1993; Lipar 2001; Verboven *et al.* 2003).

In those species where testosterone decreases with laying order (Schwabl *et al.* 1997c; Gil *et al.* 1999) or shows no overall pattern (Whittingham and Schwabl 2002; Ellis *et al.* 2001) steroid hormone production within the theca and granulosa cells may differ from that described for domestic poultry. Enzyme activity within these cells may show a different pattern as laying progresses resulting in the production of different concentrations of steroid hormones at different stages of follicular growth.

Between Clutch Patterns of Testosterone Concentration

Variation in testosterone levels between clutches in relation to environmental and social factors could be achieved by two possible mechanisms:

Increased Secretion by Follicle Cells

The rate of hormone secretion by the follicle cells could be increased. Steroid production within the ovary is under the control of LH and FSH. Increased secretion of these gonadotrophins may stimulate an increase in the production of testosterone. Gwinner *et al.* (2002) found that concentrations of luteinising hormone in female European starlings (*Sturnus vulgaris*) were greater when paired with a higher quality male. A decrease in the activity of the aromatase enzyme in the theca externa layer would also lead to an increase in testosterone levels as it is no longer being converted to oestrogen (Nelson 2000).

Plasma Testosterone

Steroid hormones move easily through cell membranes and pass into the general circulation (Nelson 2000). Therefore, blood plasma could act as the source of additional testosterone increasing yolk concentrations. Yolk is synthesised in the liver then transported via the blood to the developing follicle (Speake *et al.* 1998; Johnson 2000). It is possible that testosterone in the female's circulation is incorporated into yolk as it is transported from the liver to the follicles (Schwabl 1997b). This mechanism assumes that testosterone production in the ovary is increased and that the excess diffuses into the blood stream rather than directly being deposited in yolk (Nelson 2000).

Studies have shown that circulating testosterone levels in female birds can change in response to social stimuli. Mazuc *et al.* (2003) found a correlation between breeding density and plasma testosterone levels in the house sparrow (*Passer domesticus*). Female dunnocks (*Prunella modularis*) show elevated testosterone levels in relation to competition with other females (Langmore *et al.* 2002). Similarly, in the Bluethroat (*Luscinia svecica*) territorial competition between females caused a rise in circulating testosterone levels (Geslin *et al.* 2004).

Which Mechanism?

By examining plasma testosterone levels in relation to that found in yolk it may be possible to determine the mechanism responsible for increased yolk testosterone. A correlation between these two values would suggest that plasma is the source of the additional testosterone. However, a lack of correlation suggests that yolk testosterone levels are elevated through increased secretion by the follicle cells which diffuses directly into the developing yolk. Several authors, using both experimental and correlative methods, have examined the relationship between plasma and yolk testosterone levels.

Correlational Studies

Correlational studies have looked at the effect of an environmental stimulus on both plasma and yolk concentrations. Verboven *et al.* (2003) examined the effect of supplementary feeding on testosterone levels in the lesser black-backed gull (*Larus fuscus*). Females on the enhanced diet showed higher circulating levels of testosterone but laid eggs with lower testosterone concentrations. Similarly, a study on nesting density in the house sparrow also found raised levels of plasma testosterone and a negative correlation between maternal plasma and yolk levels of testosterone (Mazuc *et al.* 2003). In both these studies plasma samples were taken from the female after clutch completion. This may not give the most accurate indication of plasma hormone levels due to fluctuations in hormone production over the course of follicular growth as previously described.

Schwabl (1996a) measured plasma hormone concentrations in canaries breeding in differing day lengths by collecting faecal samples. This allowed the testosterone levels in female plasma to be estimated on a daily basis throughout the laying cycle presenting a clearer view of the relationship between plasma and yolk levels. Furthermore, yolk levels were compared with plasma levels from the time when the majority of the yolk was deposited in the follicle. In birds most of the yolk is deposited in the developing follicle over a relatively

short time, known as the period of rapid yolk development (RYD) (Johnson 2000). It has been estimated that in species such as the canary RYD lasts for a period of 3 days before the follicle is ovulated (Christians and Williams 2001). Schwabl's results showed a positive correlation between yolk testosterone levels and those in the female during rapid yolk deposition.

Experimental Studies

Experimental studies examining the transfer of steroids in plasma to yolk have involved administering hormones through injection or the use of implants to raise the levels in plasma. Hackl *et al.* (2003) injected radioactively labelled testosterone intramuscularly into Japanese quail (*Coturnix coturnix japonica*). Approximately 1% of the radioactive testosterone was subsequently found in the eggs. Similarly, in an experiment on zebra finches testosterone was injected subcutaneously into females following laying of the first egg (Rutkowska *et al.* 2005). Injected testosterone only accounted for 0.025% of the total amount found in the yolk of subsequently laid eggs. Dark-eyed Junco given testosterone implants before nesting laid eggs with higher levels of yolk testosterone than controls although the proportion of implanted testosterone found in the yolks was not given (Clotfelter *et al.* 2004).

The transfer of other steroid hormones has been examined. Arcos (1971) injected domestic hens intravenously with radioactively labelled estradiol and progesterone and found that the transfer to yolk represented 0.04% of the injected dose. Finally, Japanese quail were given implants containing estradiol (Adkins-Regan 1995), which produced elevated levels in female plasma and in yolk.

Aims

This study intended to examine the mechanism responsible for increased yolk testosterone levels due to mate attractiveness in the zebra finch. Female zebra finches mated to attractive, red ringed, males deposit more testosterone in their eggs (Gil *et al.* 1999). To determine whether plasma is the source of this additional testosterone, faecal samples were collected throughout the laying cycle from females paired with either a red or a green ringed male. The level of testosterone within these samples was compared to the concentration in egg yolk. Comparisons were made between data for faeces and yolk concentrations on the day each egg was laid and also over the rapid yolk development period. Yolk androgen concentrations were

compared with the accumulated total of those present in the faeces in the 3 days before ovulation.

It was predicted that if plasma is the source of additional testosterone, females mated to red ringed males would have higher plasma testosterone levels and that these levels would correlate with that found in the yolks of the eggs that they lay.

Measurement of Androgen Levels

Radioinfusion studies of birds have suggested that testosterone found in faeces is in metabolite form only (Goymann *et al.* 2002). It is not yet known which metabolites are present and in what quantities. Therefore in this study the concentration of androgen metabolites present in faeces are referred to as faecal androgens.

Androgens measured in plasma are given here as total androgen concentration. Yolk androgens are given as total androgen concentration and the concentration of dihydrotestosterone.

Methods

Birds

Female zebra finches were weighed and both tarsus and fat score (see chapter 1) measurements were taken. They were then placed in cages prepared for breeding with a nest box and nesting material. Faecal samples were collected from these females for a period of 5 days before the introduction of a male. This allowed the females to become accustomed to the sampling procedure. Following this they were paired with males wearing either red or green leg rings. All birds were kept under standard conditions as described in chapter 1.

Faeces samples were collected each day from the females until three days after clutch completion. A partition was used to separate male and female birds during the faeces collection period to ensure that samples came from the female only.

Faeces

Collection

Plasma hormone levels are known to show daily cycles (Sockman and Schwabl 1999) therefore it was important that faeces collection was undertaken at the same time each day. Between 11.30am and 12.00am plastic sheeting was placed on the floor of one half of each cage. Females were partitioned into this side of the cage and left for a period of 2 hours.

Following this the plastic was removed and the female rejoined the male. Faeces were collected, put into a labelled eppendorph and stored at -60°C. Once a week the faeces samples were freeze-dried in batches. It was necessary to freeze samples immediately on collection then dry them as steroids in faecal samples are highly prone to degradation, probably due to the large numbers of bacteria present (Buchanan and Goldsmith 2004).

Extraction

The dried sample was finely ground using a mortar and pestle and 0.01g was transferred into a conical flask. 2.5mls of 90% ethanol were added and samples were shaken on an orbital shaker for 1 hr, after which they were vortexed and decanted into a glass test tube. The samples were then centrifuged for 20 min at 1,900g. The supernatants were removed and transferred to a second set of glass tubes and evaporated at 37°C under a stream of air. A further 1.25ml of 90% ethanol was added to the first set of tubes, vortexed and centrifuged for 20 min at 1,900g. The supernatant was removed to the second set of test tubes and evaporated. Once evaporated a second extraction was completed using dichloromethane. 4 ml of dichloromethane was added to the dried sample, vortexed and shaken on a rack shaker for 30 min. The samples were then centrifuged for 20 min at 1,900g and the supernatant removed. This was dried at 37°C under a stream of air. The extracted steroids were reconstituted in 300 ml assay buffer (0.05M sodium phosphate buffer with NaCl 0.9%, EDTA 0.5%, BSA 0.5% and sodium azide 0.01%) and vortexed before the final assay procedure.

Assay

Assays were carried out by Nicola Goodship at the University of Cardiff. Androgen concentrations were measured in the faecal extracts by direct radioimmunoassay using anti-testosterone antiserum (code 8680-6004, Biogenesis, U.K.) and [^{125}I]-testosterone label (code 07-189126, ICN, U.K.).

Validation of Faeces assay

Validation of the assay procedure was carried out to assess 1) the characteristics and limitations of the assay, 2) parallelism of extracts and assay standard and 3) accuracy and precision (Buchanan *et al.* in prep.).

Faeces and Plasma Correlation

Blood plasma and faecal samples were collected on a single day from unpaired female zebra finches. This was necessary to establish that a direct relationship exists between plasma testosterone levels and that in faeces. Faeces collection was carried out as described above. Blood samples were taken under Home Office Licence by Dr. Jeff Graves (Licence no. PPL60/3162).

Female zebra finches were housed individually in cages. Faecal samples were collected, then the birds were removed individually from the cages and taken to an adjacent room where the blood sampling took place. Sampling took no longer than 5 minutes and was closely watched by the senior animal house technician. Using a sterile needle a small puncture wound was made in the wing vein and blood (10-30µl)was collected using heparinized capillary tubes. Following sampling, the birds were returned to their cages.

Blood samples were centrifuged at 3000rpm for 6 minutes. The plasma layer was removed, transferred to an eppendorph and frozen at -20°C. Evaluation of plasma testosterone levels was carried out at Cardiff University by Nicola Goodship using an identical radioimmunoassay technique to that used for the faeces extracts.

Yolk

Collection

Eggs were collected on the day of laying and replaced with a dummy egg. They were marked and frozen at -20° C awaiting analysis.

Extraction and Assay

Yolk testosterone extractions and assay were carried out as described in chapter 2. The intra-assay coefficient of variation was 2.9% for the total androgen assay and for DHT was 2.6%. The inter-assay coefficient of variation for total androgens was 19.9% and for DHT it was 38.3%.

Statistical Analysis

Statistical analysis used correlation, regression, paired t-tests, two sample t-tests and mixed general linear models where appropriate. Analysis using mixed general linear models followed a backwards-stepwise procedure. Any biologically relevant variables and interactions were included in the initial model. Variables and interactions were subsequently

removed if their probability was greater than P=0.1. Final models were checked for 'goodness of fit' using residual plots (Grafen and Hails 2002). Normality of error was tested through examination of normal probability plots and histograms of the residuals. Heterogeneity of variance was examined by plotting the residual versus the fitted values. Statistical analysis used SAS version 8e and Minitab version 12. Data were tested for normality using Kolmogorov-Smirnov tests on Minitab and transformed where necessary.

Results

Correlation of plasma and faeces androgens

Analysis of faecal and associated plasma androgen levels showed evidence of a relationship although the correlation was not significant (r=0.268, P=0.176)(Fig. 1).

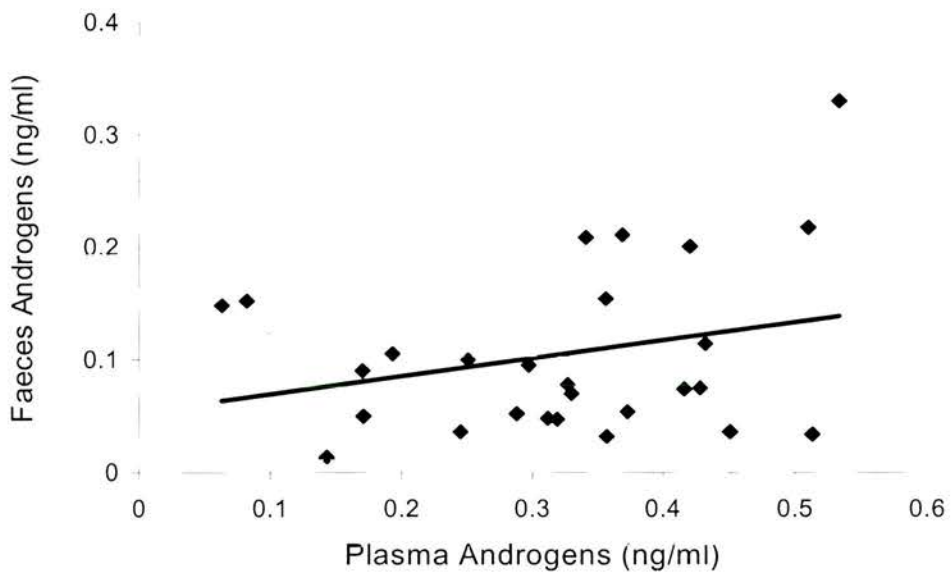


Figure 1. Regression of faecal and associated plasma androgen levels (ng/ml).

Change in Faecal Androgens at Pairing

I investigated whether pairing with a male resulted in a change in circulating androgen levels within the female. To do this, faeces were collected from the female on the day the male was added (shortly before the male was introduced) and compared to those from the day following pairing. A paired t test of all females showed no significant difference in androgen levels at the time of pairing (T=0.76, df =9, P=0.464).

To establish whether any variation in androgen levels existed at pairing in relation to male ring colour the difference between the androgen levels on the day the male was added

and the day following pairing was determined. These values were used in a two-sample t-test and showed that there was no significant difference in the change in androgen levels at pairing for females mated to red or green ringed males (T= -1.2, df=4, P=0.3)(Fig. 2).

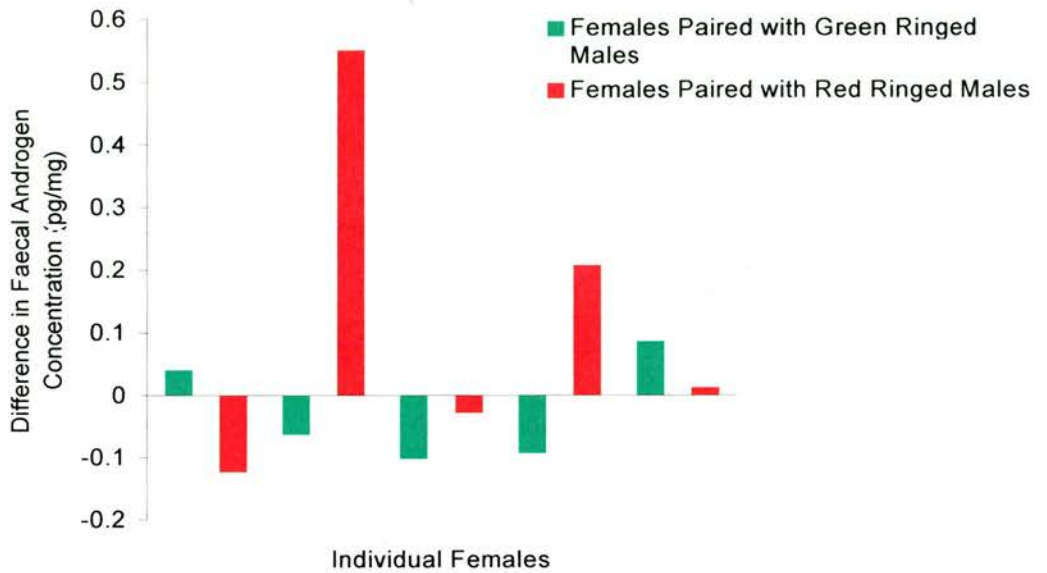


Figure 2. Difference in faecal androgen levels for individual females (n=5) before and after pairing with red or green ringed males.

Faecal Androgens across Laying Cycle

Faecal androgen levels across the laying cycle from the day before the first egg was laid (day 0) to the day following clutch completion (day 6: no females laid more than 5 eggs) were examined using a mixed general linear model. There was a significant relationship between faecal androgen concentration and day in the laying cycle ($F_{1, 55}$=7.6, P=0.0079) (Fig. 3).

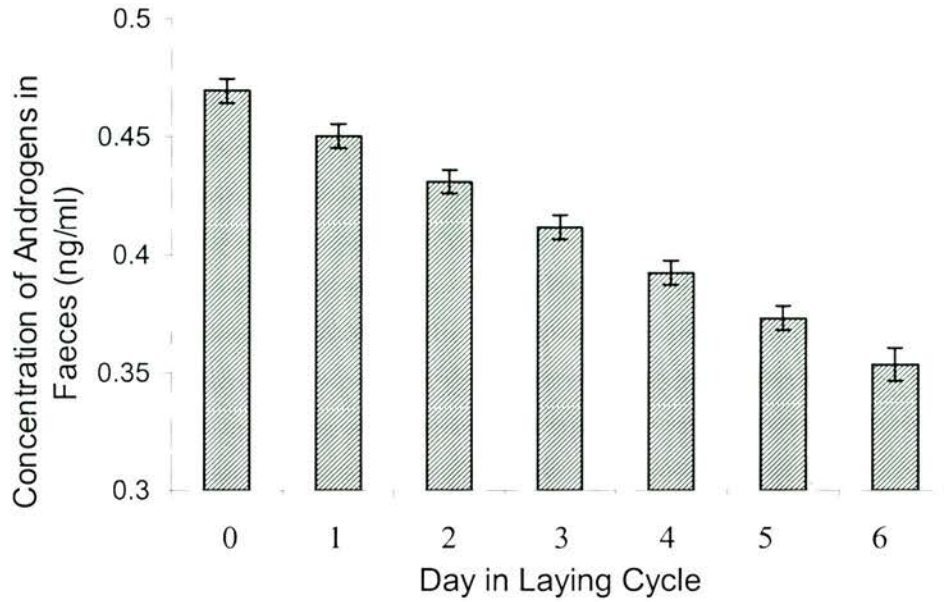


Figure 3. Fitted faecal androgen levels (ng/ml ± s.e.) across laying order.

Yolk Androgens and Laying Order

Total yolk androgen and DHT concentration with laying order were analysed using a mixed general linear model. Altogether 10 clutches were examined. Variables used were laying order, female mass and male ring colour.

Total androgens showed no relationship with laying order ($F_{1, 35}$=2.66, P=0.1117). There was a non-significant trend for DHT to decrease with laying order ($F_{1, 35}$=3.87, P=0.0570) (Fig.4).

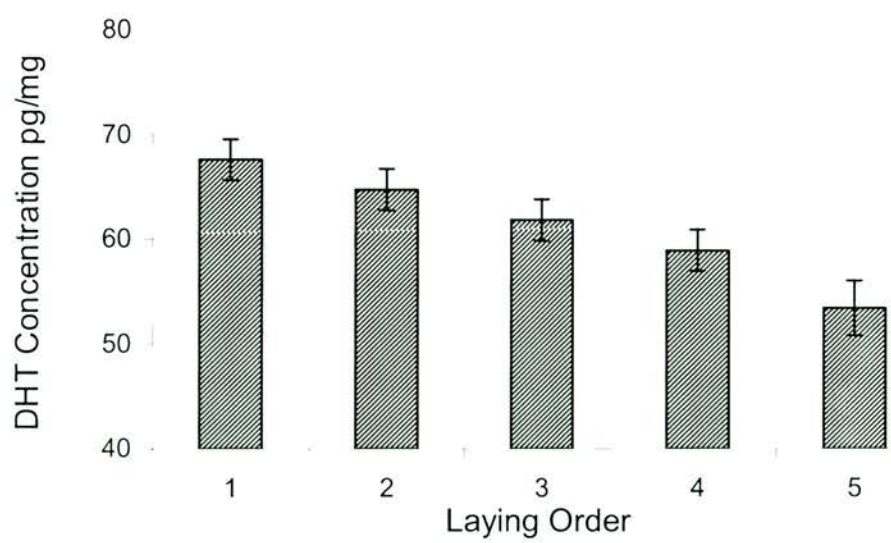


Figure 4. Fitted mean concentration of DHT (pg/mg ± s.e.) with laying order.

Faeces and Yolk Androgens

There was no correlation between the concentration of androgens in faeces and total yolk androgens (r=0.095, P=0.524) (Fig. 5), or for androgens in faeces and yolk DHT (r=0.02, P=0.899) (Fig. 6).

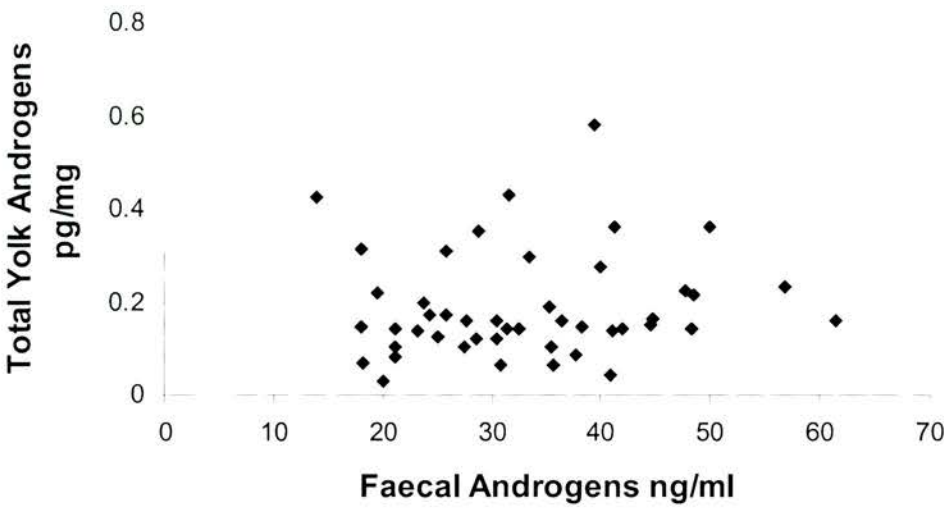


Figure 5. Total yolk androgens (pg/mg) and faecal androgens (ng/ml) from day of laying.

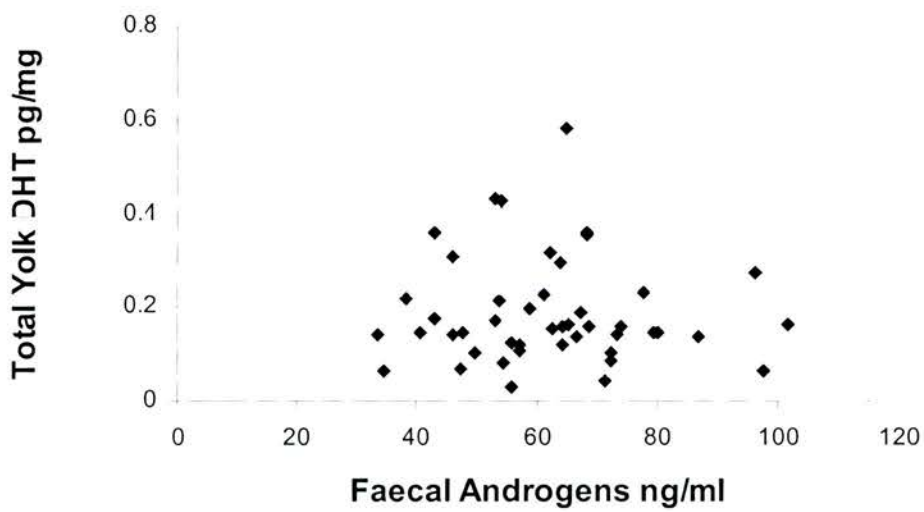


Figure 6. Yolk DHT (pg/mg) and faecal androgens (ng/ml) from day of laying.

Plasma Androgen Concentration and RYD Period

The faecal samples analysed for total androgen content were those collected one day before the first egg was laid, then daily until one day after clutch completion. To calculate values for the period of rapid yolk development it is necessary to have values from 3 days before the

follicle is ovulated. Therefore, calculating a value for the RYD period of the first laid egg would need analysis of faecal samples from 4 days before the first egg was laid. For egg 2, samples from 3 days before the first egg was laid would be needed and so on. As we analysed faecal samples from one day before the first egg was laid then it was only possible to compare the total faecal androgen concentration over the RYD period with yolk androgen concentration for eggs 4 and 5 in the laying sequence.

There was no correlation between faecal androgen concentration and the concentration of total androgens over the RYD period for eggs 4 and 5 ($r = -0.151$, $df = 15$, $P = 0.590$) or DHT ($r = 0.249$, $df = 15$, $P = 0.371$).

Discussion

In birds yolk androgen levels vary within clutches laid by different species and between clutches of the same species due to environmental and social factors. Female zebra finches lay eggs with higher concentrations of yolk androgens when mated to attractive males than when mated to unattractive males (Gil *et al.* 1999). This experiment examined possible mechanisms of increased yolk androgen deposition in relation to mate attractiveness in the zebra finch.

Androgen production in the female takes place in the ovary. Two mechanisms allowing alterations in yolk androgen levels are proposed. Androgens within yolk may originate entirely from the ovary with differences in concentration the result of changing patterns of enzyme activity involved in both the production and transformation of androgens. Alternatively, the circulatory system may act as an additional source of androgens with plasma androgens being incorporated into yolk as it passes through the blood stream from the liver to the developing oocyte. To distinguish between these mechanisms plasma androgen levels were measured across the laying cycle and compared to those found in yolk.

Androgen levels in blood plasma were measured using faecal sampling. A correlation between plasma and faecal androgen levels from a sample of female zebra finches suggested that faeces androgen levels are a reliable indicator of those in plasma (Fig.1). Of the 26 pairs of samples taken, two showed low plasma androgen levels corresponding with high faecal androgen levels. This suggests a methodology problem with the plasma samples (K. Buchanan, pers. comm.). One of these plasma samples contained fat, which can influence the levels of androgens detected. If this sample is removed from the analysis the correlation

between plasma and faecal androgen levels becomes stronger (r=0.348, P=0.082). Following removal of the second sample the correlation becomes significant (r=0.452, P=0.023).

Male Ring Colour

There was no evidence of differences in hormone production with male ring colour. Analysis of plasma levels at the time of pairing with a male showed no difference although the sample sizes here were extremely small (Fig 2). Male ring colour had no effect on the level of androgens in plasma over the laying cycle or on the concentration of androgens in egg yolk.

These results are unexpected as female zebra finches have been found to deposit more testosterone in eggs laid for attractive males (Gil *et al.* 1999). However, this may be due to the experimental design used here. Gil *et al.* (1999) used a within female, cross over design in their experiment. This would allow elimination of within female variation in yolk androgen levels, which has been shown to be substantial (e.g. Reed and Vleck 2001).

It is therefore impossible to exclude either mechanism as responsible for increased deposition of androgens in yolk with mate attractiveness. However, this study may indicate whether plasma testosterone acts as a general source of yolk androgens.

Plasma Androgen Levels

Investigation of faecal androgen levels across the laying cycle showed a pattern of significant decrease from the day the first egg was ovulated to the day after the last egg was laid (Fig. 3). This pattern was also found in studies of plasma androgen levels over the laying cycle in the European starling (Williams *et al.* 2004) and the canary (Schwabl 1996a). As far as I know these are the only studies that have examined the daily variation in plasma androgen levels across the laying cycle in non-domesticated species.

This also agrees with the pattern of androgen production by the ovary predicted by the findings of Bahr *et al.* (1983). When the full hierarchy of follicles is present and simultaneously producing hormones then the level of androgens within the ovary will be high. This is reflected in the high levels of androgens at the time the F_1 follicle is ovulated (day 0). As each follicle is ovulated fewer remain, resulting in lower plasma steroid concentrations in the ovary and a decline in the levels found in the plasma. The pattern of androgen concentration in the follicles is the opposite of this however, as each follicle accumulates androgens secreted by the others in the hierarchy resulting in an increase in yolk androgen levels with laying order. For both the European starling and the canary yolk androgen levels increase with laying order.

However, in the zebra finch yolk androgens decrease with laying order. This may suggests that the mechanism by which yolk androgens are deposited in the zebra finch is not reflected in or related to the level of plasma androgens. This might suggest that the principle source of yolk androgens is the ovary and that androgens are directly deposited in yolk.

Yolk Androgen Levels

Analysis of yolk androgen levels showed no effect of laying order on total androgen levels but there was a non significant trend for DHT concentration to decrease with laying order. Gil *et al.* (1999) found that both total androgens and DHT showed a significant decrease with laying order. This could suggest that our yolk analysis methodology may need improvement and the high inter-assay coefficient of variation for DHT in particular, suggests this.

Comparison of yolk androgen levels with those in the plasma, measured from faecal samples, showed no relationship either on the day each egg was laid or over the period of rapid yolk development. Schwabl (1996a) examined faecal androgen levels during the RYD for all eggs within a clutch for canaries and found a significant correlation.

From this it might be concluded that androgens present in yolk are not derived from plasma and it would seem more likely that they come directly from the ovary. Therefore any changes in yolk androgens are the result of changes in the enzyme activity of the ovarian cells possibly in response to changing levels of gonadotrophins.

Summary

I found no differences in the level of androgens with mate attractiveness and no relationship between levels of plasma androgens and that found in yolk. Although the study is on the whole inconclusive, it suggests that plasma is not a source of yolk androgens in the zebra finch. Further investigations with improvements to the experimental design and procedures would give a clearer indication of the source of yolk androgens. The experimental design could be improved to a cross over design where females are sequentially paired with both red and green ringed males. This would allow between female variations in androgen levels to be eliminated. I was only able to analyse enough faecal samples to look at the period of RYD for eggs 4 and 5 in the laying cycle. The faecal androgen levels for all yolks across the period of RYD should be assessed before it can be concluded that plasma androgen levels show no relationship with those in the yolk. This would give a more accurate indication of the plasma levels present at the time of yolk formation across the entire clutch.

Finally, an important point should be made regarding the study of the source of yolk androgens. As the major source of androgens in the female is the ovary, plasma androgen levels may not directly reflect androgen production in the ovary. Androgens may be directly deposited in the yolk with minimal effect to levels within the plasma. Finding a correlation between plasma and yolk androgens may therefore be misleading and researchers must be aware of this.

6

Conclusion

One of the central beliefs in life history theory is that organisms have finite resources available to invest in different aspects of their life cycle. Due to competition over these resources, their allocation to one particular trait restricts the investment that can be made in alternative traits. This results in trade-offs where an individual must choose how to partition resources in order to gain the maximum benefit (Stearns 1992; Daan and Tinbergen 1997). Such a trade off is known to exist between present and future reproductive attempts. Therefore, it has been predicted that females may be more willing to invest in offspring that give greater fitness returns at a potential cost to future reproduction and survival.

Females may increase investment in reproduction when mated to an attractive male. Burley termed this the differential allocation hypothesis (Burley 1988b). In birds, females can potentially increase their investment at several stages throughout development. By changing their behaviour or providing additional resources mothers can alter offspring phenotype enhancing offspring survival and their own lifetime reproductive fitness. These 'maternal effects' include egg composition. The objective of this thesis was to examine maternal effects due to egg composition in relation to mate attractiveness using the zebra finch (*Taeniopygia guttata*) as the study species.

Mate Attractiveness, Maternal Effects and Egg Composition

Our results showed clear evidence of the existence of maternal effects, which due to our experimental design can be attributed to egg composition. In experiment 1, females mated to red ringed males laid larger eggs than females mated to green ringed males. Chicks hatching from eggs laid for red ringed males begged more and showed greater than expected tarsus growth in relation to hatching order. For both experiments, female offspring with red ringed fathers were structurally larger as adults and laid larger eggs than females with green ringed fathers. Finally, male offspring with red ringed fathers tended to be more attractive themselves.

For the offspring of red ringed males in experiment 1, neither chick tarsus growth nor final adult size were influenced by hatching order. This suggests that chicks hatching from later laid eggs are not at a disadvantage in terms of growth as would be expected from the results of previous studies on passerines where last hatched chicks showed a slower growth rate (e.g. Lago *et al.* 2000; Johnson *et al.* 2003).

Overall our results show support for the differential allocation hypothesis as females mated to red ringed, attractive, males made a greater investment in reproduction, particularly in relation to laying order. Through this increased investment the reproductive success of the

offspring is potentially enhanced. Male offspring who are attractive themselves may be more likely to gain a mate and reproduce. By laying larger eggs, female offspring may improve the survival prospects for their own offspring. This last finding is particularly interesting as represents a trans-generational maternal effect where maternal effects from one generation are carried through to benefit offspring in the subsequent generation.

The increased investment in eggs laid for red ringed males appears to be greater in experiment 1, undertaken in the springtime, than in experiment 2, completed in the autumn. Due to these differences I decided to carry out an analysis of the effects of seasonality on clutch size and egg size with laying order for all the experiments presented in this thesis.

Seasonality in Egg Laying

Unexpectedly, when investigating the relationship between mate attractiveness and maternal effects (chapters 2 and 3), we found marked variation in maternal effects depending on the season in which the experiments were undertaken. This was despite using standardized conditions for both experiments.

A total of six experiments were carried out for the investigations presented in this thesis. Each experiment used an identical lighting and temperature regime and housing conditions were also standardized. Analysis of all these experiments together presents an opportunity to examine the effects of seasonality on clutch size and egg mass with laying order on a larger data set.

Methods

Data from all six experiments undertaken for this thesis were pooled. Statistical analysis of clutch size used a general linear model and analysis of egg weight used a mixed general linear model, with female identity as the random factor. Analysis followed a backwards-stepwise procedure starting with all relevant variables and interactions. Data were tested for normality using Kolmogorov-Smirnov tests in Minitab version 12 and statistical analysis used SAS version 8e. Suitability of the final models was determined using residual plots (Grafen and Hails 2002). Full tables of results for all variables within each model are given in the results appendix at the end of the chapter.

The effects of seasonality in the analysis were examined by classifying the experiments into 4 groups: spring (March to May), summer (June to August), autumn

(September to November) and winter (December to February). Two experiments were carried out each in spring, autumn and winter, no experiments were carried out in summer.

Results

Clutch Size

Variables used were female mass, male ring colour and season. The total number of clutches in each category was 40 for spring, 55 for autumn and 34 for winter.

Clutch size declined significantly with season ($F_{2, 122} = 6.9$, P= 0.0015) (Fig.1).

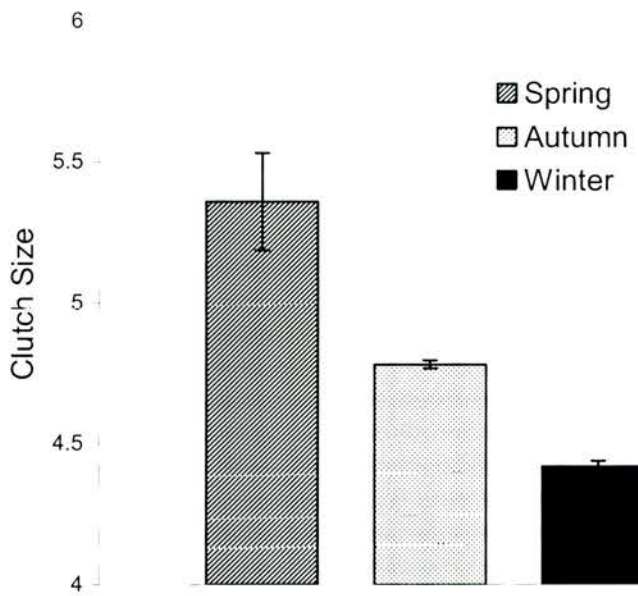


Figure 1. Fitted mean clutch size with season for all experiments.

Egg Mass

Variables used were female mass, male ring colour, laying order and season. Sample size was 600 eggs.

There was a significant interaction between laying order and season ($F_{2, 479} = 16.27$, P= <0.0001) (Fig. 2). Subsequent analysis of egg mass with laying order for each season showed that egg mass increased with laying order for spring ($F_{1, 158} = 57.04$, P=<0.0001) and autumn ($F_{1, 203} = 17.13$, P=<0.0001) but not for winter ($F_{1, 115} = 2.29$, P=0.1332).

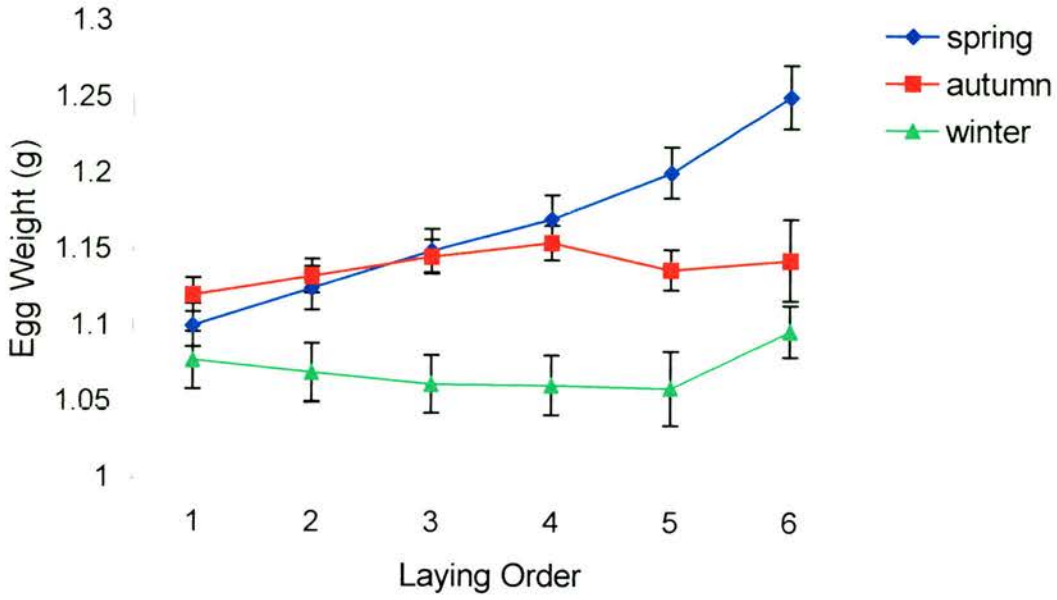


Figure 2. Fitted mean egg mass (g ± s.e.) with laying order and season for all experiments.

Females mass also had a significant effect on egg mass for all experiments together ($F_{1, 479}$=11.7, P= 0.0007)(Fig.3).

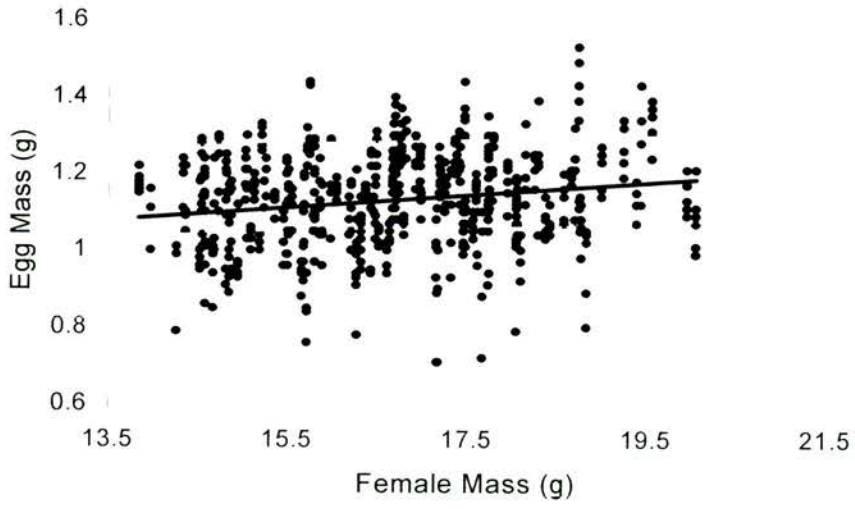


Figure 3. Female mass (g) and egg mass (g) for all experiments.

Discussion

There are clear differences in both clutch size and egg mass relating to season. Clutch size declines with season, being largest in springtime and smallest in the winter months (Fig. 1). This has been documented for other bird species including zebra finches (Zann 1996; Zann and Runciman 2003). Egg mass increased with laying order in spring and autumn but not in winter. Several researchers have documented an increase in egg mass with laying order (Rutkowska and Chicon 2005b; Royle *et al.* 2003) whereas others have not (Williams 1996; Zann 1996; Gil *et al.* 1999; Zann and Runciman 2003).

These results confirm those found previously in the two experiments completed in chapters 2 and 3, and may also help explain the differences in maternal effects with mate attractiveness that we found between the two experiments. It was evident from the results that maternal effects were greater in experiment 1, undertaken in the springtime. Chicks hatching in spring will have longer to mature and build body condition before winter, increasing the probability of over winter survival and reproduction the following season. They may therefore be more valuable to the mother, and more worthy of increased investment, than chicks hatching later in the season, which may give poorer fitness returns.

Zebra finches are widely used as a model species for laboratory based experiments, as they are easy to maintain and breed readily when given suitable conditions regardless of the time of year in which the experiments are undertaken. Despite their status as opportunistic breeders, zebra finches are influenced by photoperiod (Bentley *et al.* 2000). When housed indoors under conditions of constant temperature but changes in daylength, zebra finches show differences in egg mass and clutch size in relation to daylength (Zann and Runciman 2003). Females laid larger clutches and larger eggs under longer daylength conditions. Seasonality in reproductive behaviour in the zebra finch may therefore be an important point for other researchers to consider in the design of their experiments. This may be particularly relevant to researchers working in behavioural ecology.

Other Components of Egg Composition

Throughout this study presented in chapters 2 and 3 the emphasis has been on yolk androgens as the mediators of maternal effects due to egg composition. As a consequence of our experimental design we were unable to establish that androgens were responsible for our results, although the findings are comparable to those found in other studies on yolk

androgens. Therefore, it is not possible to eliminate the possibility that other components of egg composition may be responsible and these should be considered.

Egg size showed a greater increase with laying order for females mated to red ringed males (chapter 2 Fig. 1a.) indicated by a significant interaction between laying order and ring colour. As we have shown that hatching order does not effect chick growth then the maternal effects involved may be attributable to egg size itself or to an increase in one or all of the egg components with size or laying order.

Bird eggs consist mostly of protein, lipid and water together with a number of micronutrients including hormones, antioxidants and immunoglobulins. Experiments to examine the effects of yolk hormones on chick development have increased concentrations within yolk through injection. Experiments to determine the consequences of changes in the proportion of other egg components are mostly limited to a few examples where they have been experimentally reduced or removed. These investigations demonstrate that the removal of even small proportions of egg components has detrimental effects for the health and survival of chicks indicating that they are crucial to normal development. The effect that increases in egg constituents in potentially enhancing chick development has not been investigated.

Antioxidants

The importance of antioxidants in embryonic growth and post hatching chick survival cannot be underestimated (see chapter 4). Antioxidants are also important for immune function and the prevention of disease in adults and chicks beyond the first few days after hatching although this has been less well studied.

In chapter 4, I examined the relationship between yolk antioxidant concentration and mate attractiveness in the zebra finch. Antioxidant concentration decreased with laying order for all clutches but the pattern of decrease differed between those laid for red and green ringed males. There was little difference in antioxidant concentration between the two groups for eggs laid early in the clutch but for red ringed males later laid eggs had higher concentrations of carotenoids and alpha tocopherol.

We found evidence that growth and final size of chicks with red ringed fathers is not compromised by laying order. Therefore, given that females mated to red ringed males deposit greater concentrations of antioxidants in later laid eggs it is possible that our findings may be due to increased antioxidant concentrations in later laid eggs enhancing the development of later hatching chicks. However, the only study to examine the effect of

increased yolk antioxidant concentration on chick growth found no effect on growth (Saino et al. 2003) although immunocompetence was enhanced.

Lipids

Lipids constitute 33% of the total weight of yolk (Burley and Vadhera 1990). Yolk lipids are a vital resource. They provide the majority of the energy required by the growing embryo and are an important source of constituents for biological membranes and the precursors of some hormones and eicosanoids. About 80% of the yolk lipid is incorporated into the growing chick before hatching; the rest forms the residual yolk, which is used during the first few days of life.

Finkler *et al.* (1998) experimentally removed 20% of the yolk from the eggs of domestic chickens (*Gallus gallus*) and monitored embryo growth. Embryonic development was not affected and the only apparent consequence was a reduction in the residual yolk reserves. Female lesser black-backed gulls (*Larus fuscus*) that were induced to lay extra eggs produced later laid eggs with a significantly lower lipid content but greater water content compared to controls (Nager *et al.* 2000). The hatching success of these eggs was not significantly different from that of controls, although chicks hatched in poorer condition. Conversely, survival to fledging was significantly less for chicks from reduced lipid eggs with most mortality occurring within the first few days after hatching.

These studies indicate that reduced levels of yolk lipid do not affect embryonic growth but evidently have consequences for chick health following hatching possibly due to diminished reserves of residual yolk. On hatching the immune, digestive, nervous and endocrine systems of the chick are immature (Surai 2002). Development of these systems uses lipids stored as residual yolk as well as those that have become incorporated in the liver during embryonic growth. Residual yolk also acts as the preliminary source of nutrients as the digestive system is initially not able to absorb lipids.

Lipids may also influence chick development in more subtle ways through slight variations in yolk lipid profile (Royle *et al.* 1999). Comparisons between wild and captive bird species have suggested that maternal diet largely dictates the proportion of individual lipids present in yolk (Speake *et al.* 1999). The lipid profile of yolk affects embryonic growth and hatching success (Surai 2002), but the consequences for development beyond hatching and into adulthood have yet to be investigated.

Water and Protein

Albumen consists of 88% water with the remaining volume being mainly protein (Burley and Vadhera 1990). Due to this high proportion of water, studies that have examined the consequences of changes in the relative proportion of albumen for chick development have failed to distinguish whether any effects found are due to a reduction in water or protein.

Finkler *et al.* (1998) looked at the effect that removing albumen had on chick growth and survival. Experimentally removing 20% of the albumen from domestic chicken eggs resulted in decreased tarsus length and a reduction in water component of hatchling mass. Hill (1993) also removed albumen from the eggs of domestic fowl in proportions ranging between 1 to 16%. She found that as the proportion of albumen removed increased, hatching success and hatching mass decreased. In terms of chick growth, female chicks appeared more vulnerable. Female chicks from the albumen reduced group were significantly smaller at 20 days old than controls, whereas there were no differences between male chicks from the two groups.

Costs of Differential Allocation

The differential allocation hypothesis (Burley 1988b) assumes that there is a cost to the female in investing more resources when mated to an attractive male. Without this cost there would be no restriction on females making an increased investment with every breeding attempt (Sheldon 2000).

Egg laying is costly for birds (Monaghan and Nager 1997; Monaghan *et al.* 1998; Nager *et al.* 2001). Although these costs are apparent at several levels, most research has investigated the obvious resource based costs (Williams 2005). For example, egg production is energetically demanding (Vezina and Williams 2002; Williams and Ames 2004). The nutrients needed for egg formation may come from increased dietary intake or the use of body reserves. Depletion of body reserves can have consequences for the health and condition of the laying female (Nager *et al.* 2001; Veasey *et al.* 2001; Blount *et al.* 2004).

Egg production has other less obvious costs- it is associated with a long-term reduction in hematocrit (Williams. 2005) and an increase in oxidative stress (Wiersma *et al.* 2004). Assuming that plasma androgen levels are related to those in yolk, then the allocation of androgens to yolk incurs a cost in terms of raised androgen levels within the laying female. Although the majority of studies investigating the costs of raised plasma androgen levels have been carried out on males many of these findings should be relevant to females. In males,

elevated testosterone causes an increase in basal metabolic rate (Buchanan *et al.* 2001), a delay or suppression in moulting which can affect over-winter survival (Nolan *et al.* 1992) and a decrease in fat stores (Wingfield *et al.* 2001). Reproductive success may be lower due to reduced parental care (Hegner and Wingfield 1987), although this cost may be offset by an increase in extra-pair copulations (Raouf *et al.* 1997) or by compensation by the female (Saino and Moller 1995). In territorial species, higher testosterone levels may also result in greater injury and reduced survival due to increased aggression (Dufty 1989; Moss *et al.* 1994).

A limited number of studies have investigated the effect of elevated testosterone on females. In two studies, free-living females were given testosterone implants before breeding (Veiga *et al.* 2004; Clotfelter *et al.* 2004). Spotless starling females (*Sturnus unicolour*) with testosterone implants took longer to initiate laying and raised fewer fledglings than control females, although there were no differences in clutch size or egg mass between the two groups (Veiga *et al.*2004). Clotfelter *et al.* gave testosterone implants to dark-eyed junco (*Junco hyemalis*) females and also found delayed laying and no differences in clutch size or egg mass, nor in incubation and nest defence behaviour.

Breeding female zebra finches were injected subcutaneously with testosterone on the day after the first egg was laid (Rutkowska *et al.* 2005a). Testosterone injections were given over a range of doses and it was found that clutch size reduced with increasing testosterone concentration. Both egg mass and yolk mass increased with testosterone dose and for testosterone implanted females there was a relationship between yolk mass and laying order.

These investigations do not overall suggest a consistent cost of high circulating testosterone levels to females. Where testosterone was injected during laying it appeared to cause greater effects (Rutkowska *et al.* 2005a). Injected testosterone may have directly interfered with the naturally occurring cycle of testosterone and other hormones within the female, causing disruption of laying. But where testosterone was given to free-living birds before breeding the effects only appear to be one of a delay in initiating laying. From the time of pairing with a mate and throughout the reproductive cycle in females there is an increase in the activity of hormone secretion and transformation processes (see chapter 5). Therefore, testosterone may have been broken down or metabolised by the action of enzymes before egg formation had begun and subsequently had little influence over the egg laying process. In the study by Clotfelter *et al.* (2004) a number of captive females were also given testosterone implants but did not breed since they were housed in female only groups. Captive birds had reduced mass, were less likely to develop a brood patch and showed suppression of moult

compared to controls. These results are comparable to those found in male birds with elevated testosterone levels (Wingfield 1984; Nolan *et al.* 1992). The question of costs to the females in allocating more testosterone to yolk undoubtedly needs further investigation.

It remains possible that elevated testosterone levels during egg laying may not cause a direct physical cost to the female herself but that elevated yolk testosterone may be both beneficial and costly to her offspring In a study of black-headed gulls (*Larus ridibundus*), chicks hatching from androgen treated eggs had lowered immunity compared to controls (Muller *et al.* 2005). This reduction in immunity may have important consequences for individuals in the wild where disease and exposure to parasites is prevalent. Therefore, a cost of higher yolk testosterone levels to the chicks is essentially an indirect cost to the female herself.

Future Directions

We have shown that maternal effects due to mate attractiveness exist at the level of egg composition. As we were unable to determine the component of egg composition responsible for these effects it would be beneficial to investigate this further. Female birds vary enormously in their reproductive characteristics. Clutch size, egg size and the concentration of egg constituents such as antioxidants and androgens vary between females on a greater scale than they do within females. Therefore, it would be crucial to use an improved experimental design in future studies that overcomes this.

Our study looked not only at chick development but continued onto adulthood finding effects of egg composition on the offspring as adults. Importantly, these maternal effects on the offspring may influence the development of the subsequent generation. Few studies, particularly in vertebrate species, have examined the intergenerational impact of maternal effects so an expansion of this study into the consequences for the grand offspring generation may be valuable.

Results Appendix

Egg Weight

Variable	df	F	p
Laying Order	1, 479	20.88	<0.0001
Male Ring Colour	1, 113	0.09	0.7639
Female Mass	1, 479	11.7	0.0007
Season	2, 479	16.27	0.4041
Laying Order*Season	2, 479	16.27	<0.0001

Clutch Size

Variable	df	F	p
Season	2, 122	6.90	0.0015
Male Ring Colour	1, 120	0.42	0.5160
Female Mass	1, 121	1.07	0.3021

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