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**REPRODUCTIVE INVESTMENT IN THE ZEBRA FINCH**

***TAENIOPYGIA GUTTATA***

Submitted for the degree of Doctor of Philosophy to the University of St. Andrews by

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## **Abstract**

Reproduction is costly; therefore females are predicted to allocate resources depending upon the perceived costs and benefits of a particular breeding attempt. Within a clutch, females are also predicted to invest in offspring according to their perceived fitness returns, which may depend upon their sex and position in the hatching sequence. This thesis examines female resource allocation in zebra finches, *Taeniopygia guttata*, in relation to changes in mate attractiveness and diet quality, with an emphasis on primary investment and differential sex allocation. Females that were given an attractive male as their first mate were found to lay heavier eggs than females that were given an unattractive male as their first mate, but there was no difference in offspring quality between treatments at any stage of development. There was also no evidence of differential sex allocation in either treatment. Diet quality had a stronger effect on resource allocation and reproductive success. Females on a high quality (HQ) diet laid heavier clutches and reared more and higher quality young than on a low quality (LQ) diet. This could have partly resulted from differences in egg size and quality. Eggs laid on the HQ diet were better provisioned with yolk and albumen and had higher concentrations of yolk androgens. In line with sex allocation theory, female-biased broods were produced on the HQ diet and male-biased broods on the LQ diet. There were also sex differences in androgen deposition between the diets, with greater concentrations of yolk androgens in male eggs on the LQ diet and female eggs on the HQ diet. Egg mass increased with position in the laying sequence on the HQ diet but not on the LQ diet, which affected within-brood patterns of post-hatching mortality. Yolk androgen and carotenoid concentrations, however, decreased with laying sequence on both diets.

## **Chapter 1: General introduction**

### **Costs and trade-offs in reproduction**

Life–history theory is based on the assumption that trade-offs exist between various activities in an organism’s life (Hochachka 1992). Nowhere is this more evident than in reproduction, where time and energy trade-offs (both between and within reproductive events) are reflected in the enormous range of reproductive strategies found throughout the animal kingdom. One of the most basic trade-offs, in terms of reproductive investment, is that of quantity versus quality. At one extreme are animals such as the sponges whose reproductive effort is channelled into the release of thousands of gametes into the ocean while, at the other extreme are certain long-lived mammals, such as elephants, which rear a single offspring at a time and channel a large proportion of their effort into gestation, followed by a prolonged period of post-natal parental care. Between these two extremes lies a continuum of intermediate strategies, which influence both the amount and type of reproductive effort invested by an animal. All strategies, however, have the ultimate aim of maximizing the number of offspring that will survive to reproduce themselves.

### **Reproductive effort in birds**

Much of the work on reproductive trade-offs has been carried out on birds, which offer two main advantages. Firstly, avian reproductive effort can be divided up into three clearly distinguishable stages: egg production, incubation and chick rearing, each which can be independently manipulated to examine the costs involved (Monaghan & Nager 1997, Reid *et al.* 2000). Having said this, the majority of manipulation studies emphasised the costs of chick rearing (Daan *et al.* 1996, Orell *et al.* 1996). More recently, however, it has been demonstrated that both egg production and incubation carry considerable costs in relation to parental fitness (reviewed by Monaghan & Nager 1997).

The second advantage is that most birds are iteroparous (i.e. they breed more than once), which enables the trade-offs between reproductive attempts to be determined. However, these have received less attention compared to trade-offs within reproductive attempts.

Reproductive costs result in two basic trade-offs, which are applicable to the majority of avian species. Firstly, by investing in a greater number of offspring, offspring quality is reduced, in terms of their subsequent productivity and survival (Gustafsson & Sutherland 1988, Smith *et al.* 1989, Dijkstra *et al.* 1990a, Lessells 1991). Secondly, the female's own future fecundity and survival is compromised by investing heavily in the current reproductive attempt (Williams 1966, Røskoft 1985, Nur 1988).

### **Factors affecting trade-offs in birds**

Trade-offs between current and future reproductive success may be fundamentally different for short- and long-lived species, with resource allocation shifted towards the offspring in the former and towards the parents in the latter (Linden & Møller 1989). Within species, trade-offs are influenced by the relative costs and benefits of a particular reproductive episode. The costs to a female depend on factors such as territory quality and her own body condition or 'state' (McNamara & Houston 1996); generally, higher quality individuals should be prepared to (and able to) invest more than lower quality individuals (Pilz *et al.* 2003). Costs also depend on the social system in operation. In polygynous breeding systems, the female bears most or all of the cost of chick rearing, whereas male reproductive effort is channelled into attracting and copulating with females. Sperm competition has received a great deal of interest, and has behavioural and physiological trade-offs of its own (Birkhead 1987). In monogamous species, on the other hand, both parents rear the young. However, the female's investment is still usually greater than that of the male's, and so again, the sexes may have different priorities with respect to reproductive effort. This is especially true in species with high rates of extra-pair copulations, EPCs (Birkhead & Møller 1992).

The benefits of reproduction may also vary, depending on the perceived value of a mating attempt. This might be affected by the chances of offspring recruitment into the breeding population, which is in turn influenced by factors such as laying date (Newton & Marquiss 1984, Daan *et al.* 1990), and may mean that individuals are prepared to bear greater than average costs (Hochachka 1992). The value of a mating attempt may also be affected by male quality or attractiveness, which affects offspring quality if such traits are

inherited, and means that females are again prepared to step-up their investment (Burley 1988, Petrie 1993).

### **Differential allocation**

Female choice can operate at various stages of reproduction, both before and after pairing (Thornhill 1983). One of the most fundamental decisions that birds face after pairing is how much to invest in a particular reproductive attempt (Zhang *et al.* 1996). Given that the costs and benefits may vary for different females at different times, it is expected that females should be able to adjust their reproductive effort in relation to aspects of their social and ecological environment. Such flexibility in reproductive effort is referred to as ‘differential allocation’, a term originally coined to describe a female’s investment in relation to the attractiveness of her mate (Burley 1986a). In this thesis, I use the term ‘differential allocation’ both specifically to refer to the increase in resource investment in relation to mate attractiveness (Chapter 3), but also in a more general sense to refer to female investment in relation to factors such as diet quality and her own body condition. I also use this term to refer to the partitioning of reproductive effort among individual offspring within the brood. Differential allocation can be implemented at the level of the eggs (maternal effects) as well as at a behavioural level, such as chick provisioning (maternal and paternal effects). Maternal effects are of particular interest in this thesis and are sometimes interpreted as an important mechanism that allows an adaptive phenotypic response in offspring to an environmental cue perceived by the mother (Bernardo 1991, 1996, Arnold 1994).

### **Differential allocation within broods**

Within-brood differential allocation of resources is of particular interest in species where the young hatch asynchronously. Hatching asynchrony is typical of most altricial species (Stenning 1996), and is brought about by the female beginning incubation before the clutch is complete (Clark & Wilson 1981, Magrath 1990). This can lead to substantial age and size hierarchies within clutches, and may result in the subsequent starvation of the youngest, smallest chicks (Howe 1976, Clark & Wilson 1981, Magrath 1989). If all the young survive, then the youngest chicks usually fledge at lower mass and have lower post-

fledging survival (Nilsson 1995). Numerous hypotheses have been put forward to explain the evolution of hatching asynchrony. Up until the 1980s, the predominant among these was the *brood reduction* hypothesis (Lack 1947, 1954, 1968), which proposed that asynchronous hatching was an adaptive response to potential unpredictable food shortages. By creating a size hierarchy among siblings, the youngest offspring are quickly eliminated (Slagsvold 1986, Gibbons 1987). There is some support for the brood reduction hypothesis from species where hatching asynchrony is reduced when resources are plentiful (Murphy 1994a, Wiebe & Bortolotti 1994), or when females are in better condition (Sydeman & Emslie 1992, Wiebe & Bortolotti 1994). Along similar lines to the brood reduction hypothesis, is the *offspring quality assurance* hypothesis, the focus of which is on producing higher quality young. The size hierarchy means that at least some offspring are of high quality at fledging (Slagsvold 1986).

In 1981, Clark & Wilson proposed the *nest failure model* of hatching asynchrony, which suggests that hatching asynchrony has evolved to reduce the length of time during which the eggs and nestlings are vulnerable to predation or the elements. They also proposed the *hurry up* hypothesis, in which hatching asynchrony may help to advance the development of the first-hatched young. This enables the parents to utilise a declining food supply (Clark & Wilson 1981, Slagsvold 1986) and to produce independent young as early in the breeding season as possible (Nilsson 1993).

Other hypotheses focus on the energetic efficiency of chick rearing. The *peak load reduction* hypothesis proposes that hatching asynchrony spreads out the peak food demands of individual offspring (Hussell 1972, Bryant 1978), and the *sibling rivalry reduction* hypothesis proposes that, by establishing a stable dominance hierarchy, unnecessary (and energy-wasting) competition between siblings is avoided (Hahn 1981, Seddon & Heezik 1991).

There are additional hypotheses to those mentioned above, and they are not all mutually exclusive. Furthermore, it is likely that there are many selection pressures in different species for early incubation (Magrath 1990). Because hatching asynchrony may have evolved for reasons other than adaptive brood reduction, parents might be expected to have evolved mechanisms to either mitigate or enhance this hierarchy in response to environmental variability (Stenning 1996).

## **Mechanisms of differential allocation**

Birds possess several behavioural and physiological mechanisms by which they can adjust their breeding effort in relation to environmental conditions and their own breeding condition. The plasticity of these mechanisms varies between species, and between populations reflecting their individual life histories and associated selection pressures.

### *Behavioural mechanisms*

In species with bi-parental care, both parents can increase their investment in their brood by, for example, increasing the rate at which they feed their young (Burley 1986a, de Lope & Møller 1993). Although this can greatly influence the number and quality of offspring reared, such 'behavioural investment' is not investigated in this thesis. Rather, the emphasis here is on maternal effects, and in particular, the nature of the egg. Since the egg contains all the nutrients needed to sustain the developing embryo, females have the potential to influence a chick's survival prospects to a much larger extent than do males. In addition, since only females bear the nutritional and energetic cost of egg production, and in general, take on a larger share than the male in terms of incubation and chick rearing, it is females that are more susceptible to the trade-offs implicit in reproductive decisions.

### *Clutch and egg size*

Variation in clutch size represents the most obvious way of adjusting reproductive output, and there has been much theoretical and empirical work on the optimum clutch size under different environmental conditions (Lack 1947, Linden & Møller 1989, VanderWerf 1992). There is assumed to be a trade-off between clutch size and egg size (Smith & Fretwell 1974, Brockelman 1975) but, in reality, this is rarely the case. In fact, the converse is more frequently found, reflecting the ability of females in good condition to lay both more and larger eggs (van Noordwijk & de Jong 2003).

Within a species, there is generally a large amount of inter-female variation in clutch and egg size (egg volume or egg mass), reflecting both genetic and environmental influences (Mead *et al.* 1987). Clutch and egg size may be strongly related to food abundance, as demonstrated by numerous supplementary feeding studies (Arcese & Smith

1988, Nager *et al.* 1997, Ramsay & Houston 1997). Within-clutch variation in egg size is also common, although it accounts for a much smaller variation compared with between-clutch variation (Christians 2002). Egg mass frequently either increases or decreases with position in the laying sequence, and this has sometimes been interpreted as an adaptive brood survival strategy or a brood reduction strategy respectively (Slagsvold *et al.* 1984). In the former case, the last egg is relatively larger than the clutch mean and increases the competitive ability of that chick, whereas in the latter case, the last egg is relatively smaller than the clutch mean, and therefore places that chick at an even greater disadvantage.

### *Egg quality*

In addition to egg size, egg quality may vary between and within clutches. Both within a species, and within a clutch, eggs may vary in terms of their macro-composition, as well as their concentrations of antibodies, hormones, and carotenoids.

#### *a) Macro-composition*

Eggs may show variation in the relative proportions of albumen, yolk, proteins, lipids and water (Williams 1994). Variation in egg composition occurs both between and within species, and interspecific differences in egg composition have been correlated with various levels of hatching precocity among taxa (Carey *et al.* 1980, Sotherland & Rahn 1987). Within species, variation in egg composition may reflect female condition (Alisaukas 1986) or environmental conditions (Arnold *et al.* 1991), and is as likely to influence chick growth and survival as egg size (Ricklefs 1984, Carey 1996).

#### *b) Yolk hormones*

Lipophilic steroid hormones pass from the mother into the yolk during vitellogenesis (Schwabl 1993). Testosterone (T) has been shown to confer a number of benefits to chicks, such as increasing chick begging behaviour, growth rates and social dominance (Schwabl 1993, 1996a, Lipar & Ketterson 2000, Eising *et al.* 2001). However, T is also associated with deleterious effects on the immune system (Saino *et al.* 1995) and so it may be costly

to both offspring and mother under certain circumstances (Gil *et al.* 1999, Sockman & Schwabl 2000, Mazuc *et al.* 2003).

Testosterone concentration varies greatly in different species, as does the pattern of T deposition within clutches. The latter has been suggested to play an important role in influencing competitive hierarchies (Schwabl 1993) since yolk T has been found to increase or decrease systematically with position in the laying sequence in a number of species (Schwabl 1993, 1997, Schwabl *et al.* 1997, Gil *et al.* 1999, Lipar *et al.* 1999, Sockman & Schwabl 2000, Royle *et al.* 2001, French *et al.* 2001, Eising *et al.* 2001, Groothuis & Schwabl 2002, Mazuc *et al.* 2003).

Of particular interest are studies that have demonstrated flexibility in T deposition under different environmental conditions in a presumably adaptive manner. For example, androgen levels between clutches have been shown to vary depending on mate attractiveness (Gil *et al.* 1999), female age and quality (Pilz *et al.* 2003) and the degree of competition for nest sites (Schwabl 1997, Whittingham & Schwabl 2002, Groothuis & Schwabl 2002).

### *c) Carotenoids*

Carotenoids, which give the yolk its yellow colour, are synthesised by plants, certain bacteria and fungi, and thus are obtained from the diet (Blount *et al.* 2000). They are beneficial since they function as antioxidants, 'mopping up' free radicals and so protecting molecules such as DNA from oxidative damage. They may therefore play an important role in the regulation of immune function, and may counteract the immunosuppressive effects of T (Royle *et al.* 2001). Laying birds transfer carotenoids and other antioxidants into egg yolk either directly from the diet or from endogenous reserves (Blount *et al.* 2000). They are then transferred from the yolk to the embryo during the last few days of embryonic development (Surai & Speake 1998). Because they are a limited resource, females may transfer carotenoids to eggs differentially in relation to the reproductive value of the offspring. Alternatively, females may differentially deposit carotenoids in relation to offspring vulnerability (Saino *et al.* 2002).

### **Differential sex allocation**

Under certain circumstances, it might pay females to differentially invest in sons and daughters. Sexual allocation theory is based on the premise that males and females have different fitness returns under different environmental conditions (Trivers & Willard 1973, Charnov 1982). This is particularly evident in polygynous species, where males have much greater variance in reproductive success than females, and other species with pronounced sexual size dimorphism, where the larger sex may be more costly to rear (Myers 1978). Differential sex allocation may involve directing more resources towards one sex. For example, differences between eggs containing male and female embryos have been reported in a number of species with respect to egg size (Mead *et al.* 1987, Anderson *et al.* 1997, Cordero *et al.* 2000, 2001), hormonal composition (Petrie *et al.* 2001, Müller *et al.* 2002) and position in the laying sequence (Bortolotti 1986, Bednarz & Hayden 1991, Albrecht 2000, Badyaev *et al.* 2002). Furthermore, in nestlings, greater feeding of one sex than the other may occur (Stamps *et al.* 1987, Yasukawa *et al.* 1990, Droge *et al.* 1991, Nishiumi *et al.* 1996).

### **Sex ratio adjustment**

Alternatively, or additionally, differential sex allocation might involve differential production of male and female offspring. Efficient sex ratio adjustment is well documented in taxa such as the Hymenoptera, which have a haplodiploid sex determining mechanism. A female can exert precise control over the sex of offspring by deciding whether or not eggs are fertilised (males are haploid and develop from unfertilised eggs, whereas females are diploid and develop from fertilised eggs). In parasitic wasps, for example, only a single wasp develops per host. Since females benefit more from a larger body size, females are laid on larger hosts, and males on smaller hosts (Charnov *et al.* 1981).

However, in vertebrates where sex is chromosomally determined, this is thought to present a major constraint to sex ratio manipulation (Williams 1979, Charnov 1982). It is one of the reasons why perhaps sex ratio skews are rarely observed, but there are also other reasons such as the greater complexity and reduced predictability of the vertebrate

environment compared with that of many invertebrates (Sheldon 1998, West *et al.* 2000, Komdeur & Pen 2002).

### **Major hypotheses of sex ratio adjustment in birds and mammals**

In 1930, Fisher proposed that there should be equal parental expenditure on sons and daughters, and thus frequency-dependent selection would tend to restore an equal sex ratio. Leading on from this, when males and females are equally costly to rear, the population's sex ratio should be 1:1, but when the sexes differ in rearing costs, the sex ratios should be biased towards the sex that incurs the least amount of expenditure (Fisher 1930).

However, Fisher's prediction applies at the level of the population and not at the level of individual tendencies to skew the sex ratio. Trivers & Willard (1973) developed the *maternal condition* hypothesis based on a polygynous mammalian mating system. They proposed that parents in good condition should invest most in the sex that derives the greater increase in reproductive value from a given level of investment. In a system where males have higher variance in reproductive success than females, females in good condition should produce sons, since a high quality male will produce many offspring of his own, thus increasing the female's inclusive fitness. By contrast, a female in poor condition should produce daughters, since a poor quality daughter has a higher chance of breeding than a poor quality son.

Support for the maternal condition hypothesis later came from red deer, *Cervus elaphus*, on the Isle of Rhum (Clutton-Brock *et al.* 1984, 1986). Long-term reproductive data revealed that the lifetime reproductive success of sons was influenced much more by maternal dominance rank than was the lifetime reproductive success of daughters. This was because high-ranking mothers had heavier body weights than low-ranking mothers. Also, birth weights of offspring of high-ranking mothers were heavier than those of low-ranking mothers, and this initial inequality disproportionately affected male reproductive success. Consequently, high-ranking mothers tended to produce more sons, and low-ranking mothers tended to produce more daughters.

In contrast to the situation in red deer, studies on several primate species have found that high-ranking mothers tend to produce daughters and low-ranking mothers produce sons. The explanation in this case is tied to the inheritance of maternal rank

(Simpson & Simpson 1982, Silk 1983). Daughters, who remain in their natal group, inherit their mother's social rank, and this affects their chances of breeding. Sons, on the other hand, disperse from the group, and their rank and subsequent reproductive success is dependent on alliances formed with other males. This is sometimes referred to as the *advantaged daughter* hypothesis.

Another variant of the maternal condition hypothesis is the *attractiveness* hypothesis, which proposes that a female mated to an attractive male should produce sons. This is because sons will benefit more from inheriting genes for attractiveness and increased viability than will daughters. The framework for this hypothesis came largely from Burley's work on zebra finches, *Taeniopygia guttata* (discussed in detail in Chapter 3), but found subsequent backing from correlative and experimental studies on other avian species (Ellegren *et al.* 1996, Svensson & Nilsson 1996, Kölliker *et al.* 1999, Sheldon *et al.* 1999).

Two other hypotheses that have gained support in the last 20 years are the *local resource competition* and *local resource enhancement* hypotheses, which both stem from differences in philopatric and helping tendencies between the sexes. Because the philopatric sex competes more for resources, Clark (1978) proposed that females should invest more heavily in the dispersive sex. However, in species with helpers, it would be beneficial for females to overproduce the philopatric sex (Gowaty & Lennarz 1985). The Seychelles warbler, *Acrocephalus sechellensis*, provides the most striking example of primary sex ratio manipulation and complies well with both these hypotheses. In this cooperatively breeding species daughters are the helping sex, but they are only beneficial on high quality territories, since on low quality territories they would compete for limited insects and reduce nestling survival. Accordingly, predictable biases are observed depending on territory quality, with 77% of young produced on poor territories being sons compared to 13% on rich ones (Komdeur *et al.* 1997). By biasing the sex ratio towards males in low quality territories, breeding birds avoid having competing offspring (local resource competition hypothesis) and, by biasing the sex ratio towards females in high quality territories, breeding birds gain helpers (local resource enhancement hypothesis).

Whereas the maternal condition hypothesis emphasises the relative benefits to the offspring, an alternative hypothesis was developed by Myers (1978), which emphasised

the costs to the mother of rearing male or female offspring. It stated that only females in good condition could afford to produce the more expensive sex, whereas females in poor condition should produce the cheaper sex.

Finally, in the *brood reduction* hypothesis, females manipulate the sex ratio of their offspring within the clutch to influence the probability of brood reduction. There are numerous examples of non-random production of male and female offspring within the clutch, especially in raptor species, where there is usually pronounced sexual size dimorphism, which, together with asynchronous hatching result in pronounced competitive hierarchies (Bortolotti 1986, Bednarz & Hayden 1991).

Frequently the hypotheses are hard to disentangle, since they predict sex ratio skews in the same direction. In addition, sex ratio skews resulting from differential investment (Trivers & Willard 1973) are hard to distinguish from non-adaptive differential mortality (Clutton-Brock *et al.* 1985, Clutton-Brock 1991). The exception is when sex ratio skews are detected at conception (Nager *et al.* 2000a).

### **Mechanisms of sex ratio adjustment**

Although the above hypotheses have been applied to both birds and mammals, the physiological mechanism of sex determination is fundamentally different. In mammals, males are the heterogametic sex whereas, in birds, females are the heterogametic sex. In mammals, sex ratio adjustment at birth is thought to occur through some mechanism of sperm selection within the reproductive tract, or through hormonal influences on implantation of one sex of blastocyst (Krackow 1995). Secondly, skews could be brought about by sex-specific embryonic mortality, linked to hormone levels during pregnancy (Wise *et al.* 1985).

### **Sex ratio adjustment in birds.**

Skewed sex ratios among birds at fledging can be the result of sex ratio adjustment at ovulation (primary level) and/or differential mortality post-ovulation (secondary level). Since sex in birds is determined by the ova, which may bear either a Z or W chromosome, primary sex ratio adjustment could occur through non-random segregation of the chromosomes at the first meiotic division, or secondly, by differentially provisioning

oocytes of different sex to determine the order of release from the ovary (Ankney 1982, Krackow 1995, Oddie 1998). Post-ovulatory control could operate through sex-selective re-absorption of the ovum in the oviduct, known as atresia (pre- or post-fertilisation) or dump laying of eggs of the 'unwanted' sex (Emlen 1997). If the latter mechanism were the principle way of achieving such skews, then laying gaps would be seen, unless the primary targets were eggs at the start or end of the laying sequence. In these cases, this would result in delayed clutch completion or a smaller clutch. After laying, adjustment of the sex ratio could occur through differential mortality of one sex either prior to hatching (Blank & Nolan 1983, Rutkowska & Cichon 2002) or post-hatching. Differential mortality post-hatching has been well documented under harsh conditions when the more vulnerable sex experiences greater mortality. This is usually the larger sex which requires a greater quantity of food to sustain itself and so starves first (Howe 1976, Teather & Weatherhead 1989) or, it may be the smaller sex, which it is out-competed by the larger sex (Teather 1992, Oddie 2000). Primary sex ratio adjustment is more efficient than secondary sex ratio manipulation in that it does not waste resources, but no physiological mechanism has yet been elucidated (Komdeur & Pen 2002).

The recent availability of molecular sexing techniques in birds (Griffiths *et al.* 1996) has made it possible to determine primary sex ratios at laying, and empirical evidence has accumulated suggesting that birds are capable of adjusting their brood primary sex ratio (e.g. Dijkstra *et al.* 1990b, Ellegren *et al.* 1996, Svensson & Nilsson 1996, Heinsohn *et al.* 1997, Komdeur *et al.* 1997, 2002, Nager *et al.* 1999). However, until a physiological mechanism is elucidated, it is not clear exactly when primary sex ratio adjustment is beneficial, and how common it may be.

**The study model: the Australian zebra finch *Taeniopygia guttata castanotis***

Zebra finches belong to the subfamily Estrildinae (comprising 124 species), family Passeridae. Estrilidines are the only granivorous group of passerines endemic to Australia, the zebra finch being the most widespread and numerous (Goodwin 1982).

The Australian zebra finch is found over 75% of mainland Australia, and has reached a number of islands within 60km of the mainland coast. Its habitat requirements are accessible surface drinking water, grass seeds for food, and suitable bushes and shrubs

for nesting and roosting. Providing these requirements are met, zebra finches have proved fairly adaptable and have increased their numbers dramatically in agricultural areas (Zann 1996).

They are a highly social species, feeding and breeding in large mobile colonies of up to 50 pairs. The size of these colonies varies throughout the year depending on breeding activity and dispersal rates (Zann 1996). Zebra finches are monogamous with a strong, permanent pair bond (Immelman 1965, Zann 1995). Pairs remain in close contact within the colony and synchronise movements with constant calling to one another. These strong pair bonds enable them to begin breeding as soon as conditions become favourable, i.e. following rains which promotes ripening of grass seeds (Zann 1994a, Zann *et al.* 1995). Females also possess physiological adaptations for breeding in their unpredictable environment. The ovary of the zebra finch does not fully regress after breeding, but is maintained in a semi-developed state in which rapid follicular growth can begin more quickly than if the gonad had totally regressed. This ensures that females can breed rapidly in response to rainfall (Zann 1996).

### **Mate choice**

Both sexes incubate the eggs, and brood and rear the young (Morris 1954, Burley 1988) and as a consequence, both males and females are discriminating when it comes to choosing a mate (Burley 1985, Burley & Coopersmith 1987, Wynne & Price 1993). In multiple choice-chamber tests, males and females discriminate quickly and consistently among potential partners, although in aviary studies, it is the female that generally determines the final choice of mate (Clayton 1990). Several male characteristics have been identified as being attractive to females, such as a deeper red beak colour (Burley & Coopersmith 1987), a high display rate (Ten Cate & Mug 1984) and, specifically, a high song rate (Ratcliffe & Boag 1987, Houtman 1992, Balzer & Williams 1998). Beak colour and song rate are correlated, and condition dependent (Houtman 1992, Birkhead *et al.* 1998) but, when beak colour was artificially manipulated, it was found that females only preferred males with a high song rate, and that beak colour no longer had an effect (Collins *et al.* 1994). Furthermore, the quality of the song itself has been shown to be important. Females prefer longer songs containing a greater number of elements (Clayton & Prøve

1989). Males prefer females with orange-colour beaks (Burley & Coopersmith 1987). They also strongly prefer to mate with more fecund females, as demonstrated by supplementary feeding experiments (Monaghan *et al.* 1996, Jones *et al.* 2001).

Unexpectedly, Burley *et al.* (1982) discovered that mate choice could be strongly influenced by fitting individuals with coloured plastic leg rings. Females preferred males with red leg rings and avoided males with green leg rings, whereas males preferred females with black leg rings and avoided females with light blue leg rings. This has since become an important manipulative tool for looking at differential allocation in zebra finches in relation to attractiveness (Burley 1981, 1986a, 1986b, Zann 1994b, Gil *et al.* 1999, Zann & Runciman 2003, this thesis Chapter 3).

### **Sperm competition**

In a wild colony of zebra finches, extra-pair paternity (EPP) accounted for only 2.4% of offspring (Birkhead *et al.* 1990), although EPP was higher in an aviary study of domesticated zebra finches (Birkhead *et al.* 1989, Burley *et al.* 1996). There is last male precedence of sperm in this species (Birkhead *et al.* 1988, 1989, Birkhead & Møller 1992), and males in the wild have been observed to safeguard their paternity by both mate guarding and copulating frequently. However, it is thought that females may be able to control the frequency and timing of EPCs despite the constraints of mate guarding and the limited opportunities for extra-pair matings (Zann 1996). Rates of EPPs probably vary with the location, and will be greater in areas where the breeding season is more prolonged and therefore less synchronous, compared with arid areas where there is higher synchronicity.

### **Breeding biology**

Spherical, domed nests are constructed within thick, thorny bushes (Zann 1996). Females lay a modal clutch size of five eggs, usually beginning within a week of pairing. Both the male and the female incubate the eggs for 13 days after which time the young hatch. In the wild, the young usually hatch over a couple of days, although in the laboratory, hatching asynchrony is usually more pronounced. Both parents also feed the young, through regurgitation of seed and water from their crop. The young fledge at approximately 20

days and become independent at around 35 days. At 65 days they are sexually mature and if conditions remain favourable then they may breed that same season (Zann 1996).

### **Aims of the thesis**

This thesis investigates flexibility in female reproductive effort in zebra finches in relation to changes in their environment. Reproductive investment can be partitioned into egg production, incubation and chick rearing, and the success of a particular breeding attempt will depend on the individual optimization of allocation of effort between these phases (Heaney & Monaghan 1995). Investment in chick rearing and incubation in zebra finches have been investigated elsewhere (Burley 1986a, Heaney & Monaghan 1995, Reid *et al.* 2000, Gorman & Nager 2003). This thesis focuses on allocation of resources during egg production both between and within clutches. In birds, all embryonic development takes place in the egg, the characteristics of which are determined at laying, and affect the subsequent growth and survival prospects of the chick (Williams 1994). Furthermore, egg production is costly in terms of energy expenditure (Ward 1996), muscle condition (Houston *et al.* 1995, Monaghan *et al.* 1998), female survival (McCleery *et al.* 1996), and subsequent breeding success (Heaney & Monaghan 1995, Monaghan *et al.* 1998). Therefore, egg production presents an obvious target for differential allocation of resources.

The first part of this thesis is concerned with female reproductive investment in relation to perceived mate quality. Many avian studies have demonstrated that females prefer to mate with more attractive males (Kempnaers *et al.* 1992, Lifjeld & Robertson 1992) and have evolved a host of behavioural and physiological, pre- and post-copulatory mechanisms, to help ensure that their eggs are fertilised by sperm from high quality males (Birkhead & Møller 1993). Cues of male quality include song, plumage, and body size, although in some cases, no particular cues have been identified (Kempnaers *et al.* 1992).

Females may directly benefit from mating with an attractive male, in circumstances where male characteristics reflect parenting attributes (Norris 1990a), although in many species, attractive males may provide less care (Burley 1988, Saino *et al.* 1999, Grindstaff *et al.* 2001, Mazuc *et al.* 2003). Since attractiveness generally correlates with superior survival (Sheldon *et al.* 1999), females may also obtain indirect (genetic) benefits from

mating with an attractive male (these are the only benefits in lekking species and in the case of EPCs). Females are expected to invest more of their resources in their brood when mated to a relatively attractive male, since these offspring will be of higher quality and therefore 'worth' the extra cost of this investment.

In addition, if by mating with an attractive male, a female's offspring inherits genes for attractiveness or increased viability, the sons of attractive males might be of higher reproductive value than will daughters of such males. Therefore, a female should produce more sons when mated to an attractive male and more daughters when mated to an unattractive male. This was indeed found to be the case in two aviary experiments in which mate attractiveness was artificially manipulated in zebra finches (Burley 1981, 1986b). Females mated to more 'attractive' males fed their young more, and also produced more sons at fledging. However, in order to eliminate the possibility that assortative mating was responsible for such a result, it was decided to further investigate this work with some important experimental modifications. The use of individual breeding cages rather than large aviaries excluded free mate choice, and permitted the implementation of a paired crossover design, important in detecting changes in individual differential allocation behaviour, especially with respect to sex ratio adjustment (Oddie & Reim 2002). In addition, by using molecular sexing techniques, sex ratio (and sex-biased mortality) could be ascertained at laying, at hatching and at fledging to understand more about the timing of any observed sex ratio skew.

The second part of the thesis is concerned with the effects of diet quality on female investment. Diet is known to have a large effect on reproductive output (Houston *et al.* 1983, Bolton *et al.* 1993, Potti 1993, Smith *et al.* 1993, Wendeln & Becker 1999). As well as increasing the number and quality of young that the female is capable of rearing, a high quality diet reduces the costs incurred (Hochachka 1992). The three experiments presented in this thesis (Chapters 4, 5 and 6) were designed to investigate the flexibility of various aspects of reproduction in zebra finches in relation to diet quality and thus gain a greater understanding about the potential trade-offs that might be operating.

As well as looking at overall investment in the brood, the experiments also examined within-brood partitioning of resources. Since hatching asynchrony facilitates brood reduction of the youngest chicks, females should mitigate the affects of hatching

asynchrony when conditions are favourable. Therefore, females were expected to invest relatively more resources in the latter part of the clutch on a high quality diet compared with on a low quality diet.

Finally, sex ratio manipulation was also predicted under different diet regimes, since female zebra finches are known to be more vulnerable to poor conditions (Zann & Runciman 1994, de Kogel 1997, Bradbury & Blakey 1998, Kilner 1998, Birkhead *et al.* 1999). Three experimental studies (Bradbury & Blakey 1998, Kilner 1998, Rutkowska & Cichon 2002) have specifically investigated the effects of diet on sex ratio adjustment in laboratory zebra finches, but with competing ideas concerning the interpretation of the results.

By investigating two aspects of a zebra finch's environment (mate attractiveness and diet quality), it was hoped to gain greater insight into the selection pressures operating in this species, and also bring together some of the previous work and inconsistencies reported on this classic laboratory study model.

## **Chapter 2: General methods**

### **Experimental birds**

The zebra finches used in the experiments were all phenotypically wild-type and had all been bred in captivity. They came from a variety of sources: St. Andrews University, other U.K. universities and private U.K. breeders. It was discovered early on that naïve birds had relatively poor breeding success, and so the decision was made to use birds that had undergone at least one reproductive attempt in all experiments.

### **Housing and feeding**

Stock birds, when not being used for breeding, were kept in single sex groups on a diet of mixed seed (foreign finch mix supplied by Haith's, Cleethorpes, Lincolnshire, U.K.) containing white, Japanese, yellow, panicum and canary millet, supplemented weekly with Haith's egg biscuit and fresh spinach. All cages contained cuttlebone, oystershell grit and drinking water *ad libitum*.

Breeding birds (except for those in the diet quality experiments, Chapters 4, 5 and 6) were given egg biscuit and fresh spinach twice a week during egg laying and incubation, and three times a week when rearing chicks. Breeding cages measured 75 x 40 x 40 cm, and open-design nest boxes were attached to the front bars, which facilitated regular checks for eggs and chicks. Nesting material was provided and all birds were maintained on a 16 hr light: 8 hr dark lighting schedule with full-spectrum lights.

### **Assigning laying and hatching order**

Nest boxes were checked every morning, between 09:00 and 10:00. Newly laid eggs were weighed to the nearest 0.01g on an electronic balance, and numbered with a non-toxic pen. If no new egg was laid over two days, the clutch was considered complete. Females that failed to lay eggs within 21 days of pairing were classified as non-breeders. Nests were checked for hatchlings from thirteen days after the first egg had been laid. To ascertain hatching order, nests were inspected four times a day. In cases where two chicks had hatched since the last inspection, dampness of down was used to assess hatching order. Chicks were individually marked with coloured Tippex on their left or right leg, and this

was renewed as necessary. Between 7 –10 days chicks were fitted with orange, plastic, numbered leg rings.

### **Measurements of chicks**

Chicks were weighed as soon as possible after hatching to the nearest 0.01g. Tarsus length was also measured to the nearest 0.1mm using digital callipers. The average of three tarsus length measurements was recorded. Chicks were also weighed and measured at fledging and at adulthood (120 days).

### **Sexing**

To ascertain the sex of each egg laid, all offspring that survived to sexual maturity were sexed from plumage characteristics. Chicks that died prior to sexual maturity, and eggs that were fertile but failed to hatch were sexed from DNA extracted from tissue.

### **DNA extraction**

DNA from chick and embryonic tissue was extracted as follows: 10-20mg of tissue was digested in 600µl Cell Lysis Solution (0.1M EDTA, 0.2M Tris pH 8.5, 1% SDS). The tissue was homogenised, before adding 5µl 20mg/ml proteinase K, and incubating at 55°C over night. 3µl 10mg/ml RNase A was then added to the mixture, incubated at 37°C for 30 minutes, and subsequently cooled to room temperature. 200µl 5M Potassium acetate was added to the tube, which was then vortexed vigorously for 20 seconds. It was centrifuged at 13000 r.p.m. for 5 minutes, and the supernatant containing the DNA was decanted into 600µl 100% isopropanol. After mixing, the sample was then centrifuged for a further 5 minutes. The supernatant was poured off, and the tube drained on clean, absorbent paper. The pellet was washed with 600µl 70% ethanol, before being centrifuged for 1 minute. The ethanol was poured off and the tube drained for 20 minutes. The pellet was re-suspended in 50µl H<sub>2</sub>O and left at 4°C overnight to fully dissolve.

### **Molecular sexing**

A fragment of the W-linked avian 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 50mM MgCl<sub>2</sub>, 1µl Bioline 10xNH<sub>4</sub> reaction buffer, in a total volume of 10µl. Reactions were performed with the following thermal profile: initial denaturation at 94°C for 1 min 30s; 30 cycles of 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).

### **Hormone extractions**

Hormone extractions and assays of testosterone (T) and 5  $\alpha$ -dihydrotestosterone (DHT) were carried out by Lucy Gilbert in the Gatty Marine Laboratory, University of St. Andrews as follows:

After separating from the whites and the embryos, whole yolks were weighed and homogenised with 0.5ml water. The androgens were extracted twice using diethyl ether, and the ether fractions separated by snap-freezing in a dry ice-acetone bath. Neutral lipids were precipitated in 2ml 90% alcohol, by leaving overnight at -20°C, and centrifuging and drying the supernatant. The dried extract was dissolved in 2.5ml assay buffer. Of this, 1.5ml was kept for androgen (T and DHT) analysis. The remaining 1ml underwent an oxidation step, which involved adding 75µl oxidation reagent (5% sodium periodate, 0.5% potassium permanganate by weight) and incubating at room temperature for 20 minutes. This oxidises T such that DHT alone remains and a DHT only assay can be conducted on the final extract. This was followed by a further two extractions, and the dried extract homogenized in 1ml assay buffer for DHT analysis.

### **Hormone assays**

Radioimmunoassays (RIAs) were conducted to analyse the total (T+DHT) and DHT content of extracted yolks. 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% (Nash *et al.* 2000). Standard curves for total (T+DHT) and DHT assays were produced using T (12.5-400pg/ml) and DHT (25-800pg/ml) standards respectively. Extraction recovery efficiencies are given in the relevant experimental chapters.

### **Statistical analyses**

Analyses of egg and chick measurements were carried out using the repeated measures mixed general linear model (GLM) procedure in the SAS system for Windows Version 8 (SAS Inst. 1986). This procedure allowed the importance of several predictor variables to be examined simultaneously in an analysis of variance or covariance. To take into account the fact that the eggs/chicks within a clutch are not independent, female identity was entered as a random factor, with eggs within the clutch as a repeated measure. In the analysis of offspring development, clutches were divided up into thirds (Table 1), so that eggs were classified as falling into the first, middle or last third of the clutch (eggs virtually always hatched in the order that they were laid). This was because, in the case of unequal clutch sizes, some authors (e.g. Krebs *et al.* 2002) consider that it is the relative position in the clutch that is important in asynchronously hatching species in determining the competitive asymmetries in the broods.

Sex ratio (proportion of males, expressed as number of males/ number of sexed eggs in a clutch) was analysed at the brood level (since the sex of chicks within the brood may not be independent) by fitting a generalised linear model, using the GLIMMIX function in SAS. The error distribution was binomial with a logit link, weighted by brood size. Sex and mortality were analysed in relation to experimental treatment and laying sequence in a logistic regression. Because the logistic regression is sensitive to very small sample sizes in some of the categories, the categories 'first', 'middle' and 'last' were used for egg number (Table 1).

In the case of all models, non-significant terms were sequentially removed, starting with the least significant interaction term. All models used a Kenward Roger correction for degrees of freedom. This sometimes gives decimals for degrees of freedom, and so they were rounded up to the nearest integer. Significance was based on F-tests and taken at the

p value of 0.05. All other statistical tests were performed with MINITAB version 13 (Minitab Inc. Pennsylvania, U.S.A.).

## **Chapter 3: The influence of mate attractiveness on reproductive investment**

### **Introduction**

#### **Differential allocation**

Given that reproduction is nutritionally and energetically costly (Burley 1988, Heaney & Monaghan 1995), a trade-off is thought to exist between current and future reproductive effort (Stearns 1992). Females are therefore expected to adjust their investment in response to aspects of their environment that influence the relative costs and benefits of a reproductive event. The perceived value of a mating attempt may be influenced by the quality or attractiveness of their mate. Females are predicted to allocate more resources (and to accept greater costs) when they breed with a relatively attractive mate. This is because a more attractive male may provide direct benefits such as superior parental care (Hoelzer 1989, Norris 1990a) or, indirect benefits such as inheritance of genes for attractiveness (Fisher 1930) or viability (Hamilton & Zuk 1982, Andersson 1982, 1986, Møller & Alatalo 1999).

Work on differential allocation of resources in relation to male attractiveness has been carried out in a number of taxa including insects (Thornhill 1983, Simmons 1987), amphibians (Reyer *et al.* 1999) and mammals (Drickamer *et al.* 2000). The function of such differential allocation of reproductive investment is two-fold (Burley 1986a, de Lope & Møller 1993). Females may obtain or retain relatively attractive mates by signalling their willingness to invest more in reproduction. Alternatively (or additionally), females may invest more in offspring fathered by attractive mates to increase the quality of such offspring. Differential allocation depends on several factors, such as an individual's expected future reproductive life span, the extent of variance in the effect of mate attractiveness on offspring reproductive value, and also the expected quality of future partners. Increased allocation will only be beneficial when the current partner is more attractive than expected future partners (Sheldon 2000).

Extensive work on differential allocation in birds was first carried out by Burley working on zebra finches, *Taeniopygia guttata* (Burley 1986a). Zebra finches are socially

Table 1. Categorisation of eggs as falling into the first (1), middle (2), or last third (3) of the clutch. One-egg clutches are not shown because they were rarely incubated and therefore were not included in the analyses.

| Clutch size | Egg number |   |   |   |   |   |   |   |
|-------------|------------|---|---|---|---|---|---|---|
|             | 1          | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| 2           | 1          | 3 |   |   |   |   |   |   |
| 3           | 1          | 2 | 3 |   |   |   |   |   |
| 4           | 1          | 2 | 2 | 3 |   |   |   |   |
| 5           | 1          | 1 | 2 | 3 | 3 |   |   |   |
| 6           | 1          | 1 | 2 | 2 | 3 | 3 |   |   |
| 7           | 1          | 1 | 2 | 2 | 2 | 3 | 3 |   |
| 8           | 1          | 1 | 1 | 2 | 2 | 3 | 3 | 3 |

monogamous, forming long-term pair bonds in the wild (Immelman 1965). Although males help with incubation and chick rearing (Morris 1954, Burley 1988), attractive males have been shown to provide less parental care in this species (Burley 1988) and, consequently, females are thought to gain genetic benefits rather than economic ones from mating with such males. Support for such indirect benefits comes from laboratory experiments demonstrating that female zebra finches prefer males with redder beaks and high song rates (Burley & Coopersmith 1987, Ratcliffe & Boag 1987, Houtman 1992), which are both condition-dependent traits (Birkhead *et al.* 1998) with a heritable component (Houtman 1992, Price & Burley 1993, Price 1996). In addition, females in the laboratory seek extra-pair copulations (EPCs) with more attractive males (Houtman 1992, Burley *et al.* 1994, 1996), although a study carried out on a population of wild zebra finches found that EPCs were relatively rare (Birkhead *et al.* 1990).

Burley *et al.* (1982) discovered that fitting individuals with coloured plastic leg rings strongly affected mate choice to the extent that they overrode preferences for natural traits in zebra finches. In choice trials, females preferred to associate with males wearing red leg rings and avoided males wearing green leg rings, whereas males preferred females with black rings, and avoided those wearing pink or pale blue leg rings. These colour ring preferences were also present among wild-caught zebra finches (Burley 1988) and, since they reflect the natural plumage characteristics of the birds, they are probably related to selection for preference for their own species (Burley *et al.* 1982).

Burley (1981, 1986a) carried out two long-term aviary studies to investigate the influence of colour rings on reproductive success. In the first study (Burley 1981) both sexes were colour-ringed and released into a single aviary. Over the seven-month course of the experiment, red-ringed (attractive) males and orange-ringed (neutral with respect to zebra finch preferences) females achieved the greatest reproductive success in terms of the number of young that survived into adulthood. In a subsequent study (Burley 1986a) two further aviary experiments were carried out in which only one sex was colour-ringed per aviary; in the banded-males experiment (BME) only males were ringed with either red, green or orange leg rings and, in the reciprocal banded-females experiment (BFE), only females were ringed with black, blue or orange leg rings. Reproductive success was measured as the number of offspring surviving at least two weeks post-fledging over the

course of the experiment (22 months in the case of the BME and 15 months in the case of the BFE). Once again, ring colour was found to influence long-term reproductive success in both experiments, with attractive individuals (red-ringed males and black-ringed females) rearing twice as many offspring as unattractive individuals (green-ringed males and blue-ringed females). Burley's interpretation of these results was that an individual's own attractiveness affected the amount of parental investment that it was able to secure from its mate. Individuals were willing to provide above-average (for their sex) parental investment to obtain and/or retain relatively attractive mates and were prepared to withstand the costs such as decreased future reproductive potential. The fitness of attractive individuals was increased by restricting their own per-offspring investment, so conserving their reproductive effort for future breeding attempts. However, because there was the opportunity for assortative mating in the aviaries, Burley could not categorically reject an alternative hypothesis: the differential access hypothesis, in which a more attractive individual is able to obtain a superior mate, and so can enhance the quantity and/or quality of offspring produced. In great tits, *Parus major*, for example, males with large breast stripes were found to mate with females that had previously laid large clutches (Norris 1990b).

To gain support for the differential allocation hypothesis, Burley (1988) analysed parental time-budgets from the BME and the BFE. Under the differential allocation hypothesis, less-attractive individuals were predicted to increase their parental investment, and more attractive individuals were predicted to decrease it. In line with these predictions, attractiveness of individuals was negatively correlated with parental investment, to the extent that some males became polygynous. Other predictions from the differential allocation hypothesis were also supported, such as lower mortality among attractive individuals compared with unattractive individuals.

Swaddle (1996) carried out an aviary study on domesticated zebra finches using symmetrically and asymmetrically arranged leg rings to create attractive and unattractive males respectively (Swaddle & Cuthill 1994). As in Burley's experiments, attractive, symmetrically-ringed males produced more offspring that survived to independence. However, since no time-budget analyses of parental activities were carried out, this result could have been brought about through either differential access, i.e. higher quality

females mating with symmetrically-ringed males or, through differential allocation, i.e. females contributing increased levels of parental investment towards offspring of attractive, symmetric fathers.

A more discriminating test of the differential allocation hypothesis is to eliminate free mate choice or to manipulate attractiveness after pairing (Sheldon 2000). The latter approach was adopted by Zann (1994b) who fitted leg rings on already-mated zebra finches in two wild breeding colonies. In one colony, he specifically tested the effects of ring colour on male and female body condition (measured in terms of differences in mass, moult and bill colour before and after breeding) and survivorship (measured in terms of recapture rates 12 months after ringing). In a second colony, data from a long-term population of colour-ringed birds were used to investigate the influence of attractive ring colours on survivorship and reproductive performance. In contrast to Burley's studies, ring colour had no effect on survivorship in either colony, nor did it have any effect on body condition. Zann suggested that this might be because breeding in the wild is less intensive compared with in the laboratory where birds were permitted to breed continuously. However, in support of the differential allocation hypothesis, Zann did find that females mated to red males laid an extra clutch. Because the majority of birds were already paired before being ringed, this increased investment could only be explained in terms of the differential allocation hypothesis, not by the differential access hypothesis.

A similar approach was adopted by de Lope & Møller (1993) who investigated differential allocation in a field study on barn swallows, *Hirundo rustica*. Female barn swallows, when mated to males with experimentally lengthened tail feathers, fed their young more frequently, and reared more young compared with females mated to males with experimentally shortened tails. Since the manipulation was performed after pairing, the increase in reproductive effort was assumed to be due to differential allocation on the part of their mates. However, the elongated tails impaired male foraging efficiency, and the result could be explained by a compensation effect (Witte 1995) rather than because females invested more than average for their sex because they had acquired an attractive partner.

More recent studies on differential allocation have focused on primary reproductive effort (i.e. at laying) for three main reasons. First, egg production is both nutritionally and

energetically costly (Houston *et al.* 1983, Heaney & Monaghan 1995, Carey 1996, Ward 1996, Monaghan *et al.* 1998). Second, intraspecific variation in investment in egg production has been shown to significantly affect offspring growth and survival in birds (Heaney & Monaghan 1995, Monaghan *et al.* 1995, Styrsky *et al.* 1999). Third, differential allocation at the egg stage is not confounded by male effort. This is in contrast to the chick provisioning stage, where females may be forced to increase their provisioning rate if attractive males invest less (Witte 1995, Qvarnström *et al.* 2000).

In peahens, *Pavo cristatus*, clutch size was positively correlated with male train size (Petrie & Williams 1993), and in mallards, *Anas platyrhynchos*, females laid larger eggs when mated to more attractive males (Cunningham & Russell 2000). Balzer & Williams (1998) investigated differences in primary reproductive effort in zebra finches. Natural male attractiveness was determined using choice chamber tests, and females were mated to an attractive and an unattractive male in a paired crossover design. Male attractiveness was found to have a positive, but weak, effect on clutch size; individual females laid on average 0.5 eggs more when paired with an attractive male compared to an unattractive male. However, there were no differences in the proportion of birds that laid eggs, the number of days between pairing and laying, mean egg size or egg quality (protein and lipid composition). Therefore, they concluded that most types of primary investment are relatively inflexible in this species.

Mate attractiveness may also affect the quality of eggs laid. Female barn swallows paired to males with experimentally lengthened tail feathers laid eggs with higher concentrations of yolk carotenoids and immunoglobulins (Saino *et al.* 2002). Using the same colour ring manipulations as Burley *et al.* (1982), female zebra finches mated to red-ringed (attractive) males were found to deposit more testosterone (T) and 5  $\alpha$ -dihydrotestosterone (DHT) in their eggs (Gil *et al.* 1999). Greater deposition of T and DHT could confer several advantages on chicks. For example, studies of canaries *Serinus canaria*, red-winged blackbirds, *Agelaius phoeniceus*, and black-headed gulls, *Larus ridibundus*, suggest that maternally-derived yolk androgens enhance chick begging behaviour, growth rates and social dominance (Schwabl 1993, 1996a, Lipar & Ketterson 2000, Eising *et al.* 2001). Gil *et al.* (1999) suggested that there might be a cost involved in T deposition incurred either by the offspring (Sockman & Schwabl 2000, Mazuc *et al.*

2003) or by the female, such that only offspring resulting from high quality mates are worth, or can withstand, this greater androgen investment (although this has yet to be tested). However, since the eggs in their study were not sexed, another possible explanation for their result is that females were laying more male eggs when mated to attractive males (see below), and that male eggs contained more T than female eggs (Petrie *et al.* 2001).

### **Differential investment in male and female offspring**

As well as influencing reproductive effort between breeding attempts, mate attractiveness may affect differential investment between male and female offspring within a breeding attempt. Sex allocation theory predicts that parents should bias investment toward the sex that confers higher relative fitness on the parents (Trivers & Willard 1973, Charnov 1982). Differences in the relative costs and benefits of rearing male and female offspring are most evident in polygynous species. In these species male variance in reproductive success is much higher than that of females and, additionally, there is usually pronounced sexual size dimorphism, with the larger sex being more costly to rear (Myers 1978). In species where variance in reproductive success is higher in males than females, females mated to attractive males should bias reproduction towards sons. This is either because, the attractive trait is heritable (Fisher 1930, Weatherhead & Robertson 1979, but see Griffith *et al.* 1999) or, because the trait indicates increased viability (Andersson 1986), and the more costly sex or the sex with the highest fitness returns will benefit more from higher quality genes (Sheldon *et al.* 1999). Either way, sons of attractive males are expected to be of greater reproductive value than daughters of such males, and the reverse would be true for offspring of less attractive males (Komdeur & Pen 2002).

Large differences in the variance of reproductive success between males and females also occur in socially monogamous species with high rates of extra-pair copulations (EPCs) (Birkhead & Møller 1992). A female benefits from EPCs with males that are more attractive than her own mate because her eggs will have an opportunity to be fertilised by high quality males, and produce offspring with genes for attractiveness or genes for general vigour. Evidence for such genetic benefits is mixed. Kempnaers *et al.* (1997) found that extra-pair young blue tits, *Parus caeruleus*, were more likely to survive

the nestling and juvenile growth periods than within-pair young. However, in tree swallows, *Tachycineta bicolor*, there was no difference in the viability of within-pair and extra-pair young prior to fledging (Whittingham & Dunn 2001) and in coal tits, *Parus ater*, there was no difference in terms of local recruitment and first year reproductive success (Schmoll *et al.* 2003).

Sex-biased allocation can occur at a number of levels; for example, there may be preferential feeding of one sex (Stamps *et al.* 1987, Yasukawa *et al.* 1990, Droge *et al.* 1991, Nishiumi *et al.* 1996). Differential investment in sons and daughters may also occur at the level of the eggs. Male eggs are larger than female eggs in the white-crowned sparrow, *Zonotrichia leucophrys oriantha* (Mead *et al.* 1987), and in the house sparrow, *Passer domesticus* (Cordero *et al.* 2000). Similarly, in leg-horn chickens, *Gallus gallus domesticus*, dominant females were found to allocate more yolk T to male eggs, whereas subordinate females allocated more T to female eggs (Müller *et al.* 2002). These patterns of differential allocation should be adaptive because, in all these species, the promiscuous behaviour of males means that they have higher condition-dependent variance in reproductive success than do females.

In both of Burley's aviary studies (Burley 1981, 1986b, see earlier for details of experimental set-ups) the sex ratio at fledging was biased towards the sex of the more attractive individual. In the first aviary experiment (Burley 1981) the skew was more pronounced because both males and females were colour-ringed, thus enabling pairings between individuals of extreme attractiveness and unattractiveness, i.e. green-ringed males with black-ringed females and red-ringed males with blue-ringed females. In the second experiment (Burley 1986b), in which only one sex was colour-ringed, the results were qualitatively similar. In the BME red-ringed males had the most male-biased sex ratio, and in the BFE black-ringed females had the most female-biased sex ratio. Although zebra finches are socially monogamous, as mentioned earlier, Burley's work demonstrated that there were substantial fitness benefits to attractive individuals; they had higher long-term reproductive success (Burley 1986a), lower parental investment (Burley 1988), and lower mortality (Burley 1985). Furthermore, in the case of males, some red-ringed males became polygynous (Burley 1985, 1988). Conversely, females mated to green-ringed males participated more in EPCs than mates of red-ringed males and performed EPCs

preferentially with red-ringed males (Burley *et al.* 1994, 1996). As such, there was an additional double benefit to red-ringed males; they had greater paternity confidence and greater opportunities for EPCs.

A sex ratio skew at fledging could have been brought about in a number of ways. In birds, females are the heterogametic sex and so have the potential to adjust the sex ratio at ovulation (primary sex ratio adjustment) through non-random segregation of the chromosomes at meiosis (Krackow 1995). Alternatively, or additionally, sex ratio adjustment could be brought about post-ovulation (secondary sex ratio adjustment) through selective re-absorption of eggs of the 'wrong' sex (Emlen 1997), dump laying of these eggs or, through differential mortality of one sex post-hatching. A sex bias in post-hatching mortality could arise through selective provisioning of offspring of one sex by the parents, or through differential vulnerability of one sex to poor parental investment or unfavourable rearing conditions (Clutton-Brock 1991).

Molecular techniques were unavailable to Burley at that time for sexing offspring that died before maturity, so the sex ratio skew found at fledging could have been brought about pre- or post-hatching. Because there was no sex ratio bias among clutches where there was no mortality, Burley suggested that primary sex ratio manipulation was unlikely. In addition, sex ratio trends for clutches with pre-fledge brood losses were stronger than, and in the same direction as, the overall trend. Therefore, Burley concluded that the skew was probably caused by post-hatching brood manipulation by the parents, possibly using sex differences in begging calls as the cue. Recently, a study by Zann & Runciman (2003) found no evidence for sex ratio manipulation at laying in relation to male attractiveness in zebra finches, but they did not investigate the possibility of sex ratio adjustment post-hatching.

In a zebra finch aviary study (with no experimental manipulations), Clotfelter (1996) found that the sex ratio was male-biased at fledging. Male zebra finches were found to feed male-biased broods more frequently than female-biased broods, but there was no measurable effect of this feeding bias on male survival. He also found that males hatched earlier within the clutch than females, although there was no evidence of increased mortality of late-hatching females. His work did suggest possible mechanisms by which parents could potentially adjust the sex ratio post-hatching.

If sex ratio adjustment is advantageous, then it is most efficient, and less wasteful in terms of resources, at laying (Komdeur & Pen 2002). However, relatively few studies have found convincing evidence for primary sex ratio manipulation in relation to mate attractiveness. Ellegren *et al.* (1996) found that collared flycatcher, *Ficedula albicollis*, pairs produced more daughters when the size of the sexually selected forehead patch of the male had been reduced by experimentally elevating his parental effort the previous breeding season, and the opposite was true for males whose parental effort was experimentally reduced. However, because they did not directly manipulate the male secondary sexual characteristic, such a correlation could also have arisen due to differences, for example, in territory quality resulting from such a manipulation. In blue tits, females mated to males that survived to the next breeding season had clutches with a significantly higher sex ratio (i.e. more males) than females mated to males that did not survive (Svensson & Nilsson 1996). In great tits, the proportion of males in a brood was correlated with male body size as indicated by tarsus length (Kölliker *et al.* 1999), although territory quality could have again been a confounding factor in this study.

The problem of confounding variables is reduced when the attractive characteristic is directly manipulated. Sheldon *et al.* (1999) masked the ultraviolet-reflecting plumage ornamentation of already-mated male blue tits with ultraviolet absorbing sun-block, and found that this reversed the positive correlation between reflectance and brood sex ratio observed in control pairs. Since ultraviolet reflectance predicted male survival to the following breeding season, this suggested it provided a cue to females of male viability.

However, there are a far greater number of published studies that have failed to find any correlation between mate attractiveness and sex ratio, either at a primary or secondary level. For example, no significant relationship was found between offspring sex ratio and male quality in the great reed warbler, *Acrocephalus arundinaceus* (Westerdahl *et al.* 1997). Similarly, in barn swallows, experimental lengthening of the sexually selected tail feathers did not lead to a difference in sex ratio (Saino *et al.* 1999). In male dark-eyed juncos, *Junco hyemalis*, Grindstaff *et al.* (2001) experimentally elevated the levels of circulating testosterone (which makes them more attractive to females) but found no difference in the sex ratio of offspring of females mated to these males compared with females mated to control males. There has also been conflicting evidence within species

and even within populations. Leech *et al.* (2001) examined how the brood sex ratio of 154 broods of blue tits related to a large number of factors reflecting parental quality, and found no significant predictors. Similarly, no sex ratio skews were detected in a five year study of breeding great tits by Radford & Blakey (2000).

An additional approach has been to examine the sex of extra-pair young. These offspring tend to be fathered by high quality males (Birkhead & Møller 1992) so it would be beneficial for the female to target these eggs for sex ratio manipulation. In blue tits, the proportion of males was greater among extra-pair than within-pair offspring (Kempnaers *et al.* 1997). However, this finding has not been corroborated by studies in red-winged blackbirds (Westneat *et al.* 1995), collared flycatchers (Sheldon & Ellegren 1996), great reed warblers (Westerdahl *et al.* 1997), barn swallows (Saino *et al.* 1999), or blue tits (Leech *et al.* 2001). This might reflect an inability by females to exert such a fine level of control over the sex of extra-pair young (Leech *et al.* 2001), which is unsurprising given that the egg is fertilised the day before laying, by which time the sex is already determined.

### **Aims**

The aim of this chapter was to investigate differential allocation in zebra finches, in the absence of assortative mating, with particular emphasis on primary reproductive effort and differential sex allocation at laying. A paired crossover experimental design was employed to investigate changes in egg resource allocation by individual females when paired with both an attractive and an unattractive mate. Attractiveness was manipulated with leg rings for which females have known colour preferences (Burley *et al.* 1982). An advantage of using an artificial ornament rather than natural male attractiveness is that any fitness benefits seen in the offspring are unlikely to be due to male quality, and must therefore be due to differential allocation by the female. Under the differential allocation hypothesis, females were predicted to lay larger, heavier clutches and deposit more androgens into yolks when mated to an attractive male. The offspring resulting from such matings were consequently predicted to be of higher quality.

Sex ratio adjustment was investigated at laying, at hatching and at fledging, the prediction being that if the fitness benefits of producing sons over daughters when mated to attractive males is substantial, then females should skew their sex ratio towards sons at

laying. The crossover design would provide a powerful test of the tendency for individual females to alter their primary sex ratio in relation to the relative attractiveness of their mate (Oddie & Reim 2002). If parents manipulated the sex ratio post-hatching (Burley 1986b), this could also be directly tested and, with knowledge of the laying sequence, a possible cuing mechanism could be determined (c.f. Clotfelter 1996).

## Methods

### Experimental design

Fifty male zebra finches were randomly assigned to one of two groups. Males in one group were made attractive by fitting a red ring on each leg, and males in the second group were made unattractive by fitting a green ring on each leg (Burley *et al.* 1982). Fifty females, which had previously had at least one breeding attempt (but not with a colour-ringed male and not within six months prior to the start of the experiment) were weighed and randomly assigned either a red-ringed or a green-ringed male with whom they had not previously bred. Females were identified by numbered, orange leg rings (which are neutral with respect to zebra finch preferences). All leg rings were supplied by A.C. Hughes, Middlesex, U.K.

Prior to pairing, male and female condition was estimated by visually scoring the amount of stored fat in the furculum between the clavicles. Scores were given from 0 to 5, such that 0 is no fat, 1 is a trace, 2 is some fat but still concave, 3 is filled but slightly concave, 4 is filled and at least level with inter-clavicles, and 5 is convex, overflowing the length of the furculum (Helms & Drury 1960). Pairs were kept in individual breeding cages, and fed on foreign finch mix, supplemented twice a week with Haith's egg biscuit and fresh spinach. Fresh drinking water, oyster shell grit and cuttlebone were available *ad libitum*. Open-design nest boxes were attached to the front of the bars, and nesting material was provided. All birds were maintained on a 16 hours light: 8 hours dark lighting schedule with full-spectrum lights. This is important as bird vision extends into ultraviolet end of the spectrum and has been shown to be important in mate choice in zebra finches (Bennett *et al.* 1996, Hunt *et al.* 1997).

Nest boxes were checked for eggs and chicks as described in Chapter 2. The second egg of each clutch was removed for androgen analysis and replaced with a dummy

egg. These second eggs were placed in an incubator at 37.5°C for 96 hours to enable an embryo large enough for sexing to develop. The embryo was removed for sexing and the yolk was separated from the albumen for androgen extraction and assay (see Chapter 2).

Nests in which no eggs hatched were given chicks from non-experimental birds to ensure that the maximum number of females had equivalent breeding experience in the second round of the experiment. Once the chicks had reached independence, chicks were removed and pairs were separated and transferred to single sex groups. One month later, females were re-paired with a different mate in the reversal round of the experiment. Females paired with red-ringed males in the first round of breeding were given green-ringed males, and vice-versa.

Analysis of the egg mass data from the first two rounds of breeding seemed to suggest a carry-over effect, whereby females were investing in a similar manner in both rounds of the experiment, despite different treatments. A third round of breeding was therefore carried out to test whether a longer interval between pairing would alter female investment. The third round of breeding was started after females had been given a two-month break since they were with their last mate, during which time birds were kept in single sex groups. In this third round, females were given a new mate, with the same colour ring as her second mate. All the eggs were removed and weighed on the day of laying and replaced with dummy eggs. The eggs were not incubated in this third round but the fresh eggs were dissected to obtain data on macro-composition. The yolk and albumen were separated, and the yolk was visually scored for carotenoid levels using a Roche colour fan on a scale of 1–15, with 15 being the darkest shade of yellow. The albumen and yolk were weighed to the nearest 0.0001g to obtain their wet mass. They were then dried to constant weight at 65°C for 48 hours, and re-weighed to obtain their dry mass.

### **Sexing**

DNA was extracted from incubated second eggs, chicks that died prior to sexual maturity, and eggs that were fertile but failed to hatch, using the method described in Chapter 2. The PCR-based sexing technique is also described in Chapter 2. Offspring that survived to sexual maturity were sexed from plumage characteristics.

### Hormone extraction and assays

See Chapter 2 for methods. The intra-assay coefficient of variation was  $4.3 \pm 0.3\%$  (mean  $\pm$  SE) for T and  $6.3 \pm 0.6\%$  for DHT. The inter-assay coefficient of variation was  $17 \pm 2.2\%$  for both. The extraction recovery of total androgens (T + DHT) was  $75.6 \pm 9.0\%$  and the extraction recovery of DHT only was  $59.8 \pm 0.9\%$ . The cross-reactivity of the antisera was 46% (see also Nash *et al.* 2000), so the T content was estimated as total-(0.46 x DHT).

### Statistical analyses

Analyses of egg mass, offspring development and egg macro-composition data were carried out using a repeated measures mixed general linear model (GLM) procedure (see Chapter 2), with female as a random factor, male ring colour as a fixed factor and egg number as a covariate. Sex ratio and mortality data were analysed using generalised linear models as described in Chapter 2. In the analyses of hatching and fledging sex ratios, and embryonic and post-hatching mortality, data were excluded from all second eggs since these were removed for steroid analysis and it was not known whether or not the embryos would have survived.

Total yolk androgens were calculated by multiplying initial yolk mass by androgen concentration. Because the development of an embryo disrupts the yolk, I estimated the initial yolk mass from the whole egg mass using the regression equations obtained from data on the composition of fresh eggs laid on a high quality diet, since eggs were of a similar mass (Chapter 6:  $y = 0.028 + 0.145 e$ , where  $y$  = yolk mass and  $e$  = egg mass). This has the advantage that the calculated yolk mass would relate to initial yolk mass (prior to embryonic development) and thus make no assumptions concerning the rate of development of the embryo, which would in turn also affect the mass of the remaining yolk.

Paired analyses for egg mass data were carried out for the first two rounds of breeding, but due to a significant interaction with order of treatment (see results), the first and second breeding rounds were also analysed separately. For data on brood sex ratios, mortality and offspring development, both paired analyses and individual analyses for each round of breeding were carried out. This was because of the order effect found for the egg

mass data, which suggested that there was a stronger effect in round one, and also because the sample sizes for paired data were small.

## Results

### Laying data

Thirty-nine out of 50 females laid a clutch with both a red- and a green-ringed male (21 females laid a clutch with a red-ringed male and 18 females laid a clutch with a green-ringed male in round one). Four females did not lay a clutch with either male, and seven females laid a clutch with only one of their given males. Of these, two laid only with the red- and five laid only with the green-ringed male.

Paired analyses were carried out for the 39 females that laid clutches with both males. There was no difference in the median number of days to laying or in the median clutch size between treatments (Table 1).

Egg mass data were analysed in a repeated measures GLM, paired for females that laid with both males (Table 2). Egg mass increased significantly with position in the laying sequence (Fig. 1) and there was a significant three-way interaction between ring colour, female body mass and breeding round with respect to egg mass ( $F_{1,37} = 6.30$ ,  $p = 0.02$ , Fig. 2). This interaction was due to females investing differently with respect to the ring colour of their mate in the first round of breeding, but not adjusting their pattern of investment in the second round of breeding when the treatments were reversed (Figs. 1 & 2).

In the first round of breeding, females mated to red-ringed males laid heavier eggs than females mated to green-ringed males (Fig. 1a). In the final model for this first round of breeding (Table 3) there was a significant interaction between ring colour and female body mass with respect to egg mass (Fig. 2a). There was a significant positive relationship between egg mass and female body mass for females mated to red-ringed males ( $F_{1,20} = 16.30$ ,  $p = 0.0007$ ), but not for females mated to green-ringed males ( $F_{1,15} = 1.99$ ,  $p = 0.18$ ). There was no difference in the mass of male and female eggs ( $F_{1,89} = 1.89$ ,  $p = 0.17$ ) and the interaction between ring colour and embryo sex with respect to egg mass was not significant ( $F_{1,88} = 3.26$ ,  $p = 0.09$ ).

Table 1. Paired laying data for females that laid clutches when mated to both a red- and a green-ringed male (n = 39 females). Data were analysed using a Wilcoxon signed ranks test.

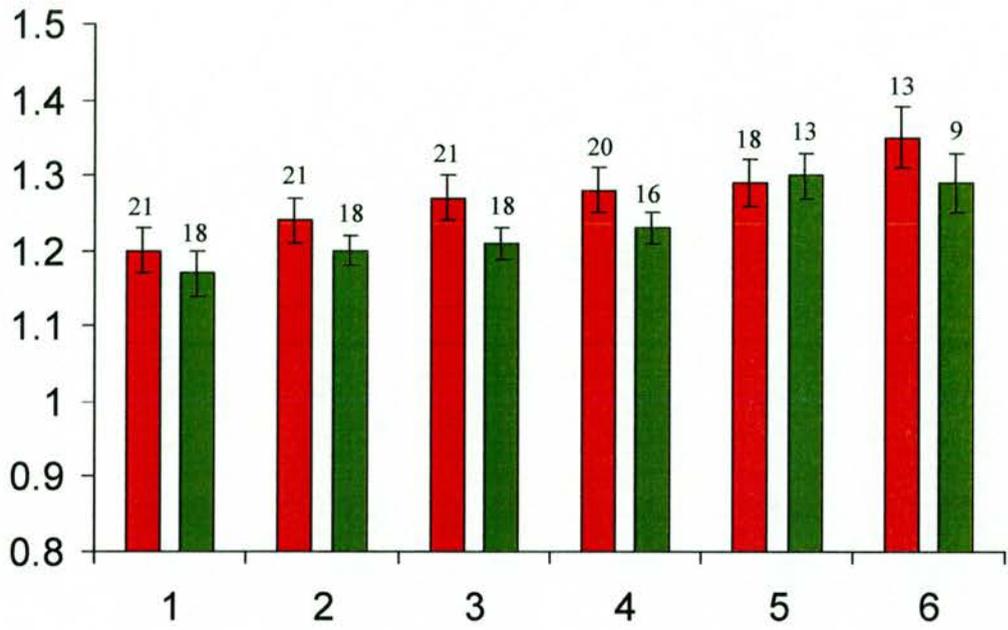
|  | <b>Red-ringed mate</b> | <b>Green-ringed mate</b> |                     |
|--|------------------------|--------------------------|---------------------|
| <b>Median no. days to 1<sup>st</sup> egg<br/>(Interquartile range)</b> | 6 (6-7)                | 5 (5-8)                  | W = 174.0, p = 0.35 |
| <b>Median clutch size (IQR)</b>  | 6 (5-6)                | 6 (5-6)                  | W = 209.0, p = 0.40 |

Table 2. Factors influencing the mass of eggs (Repeated measures GLM analysis). The data were paired for 39 females (426 eggs) that laid a clutch with both a red- and a green-ringed male. Non-significant terms were removed from the model, starting with the least significant interaction terms, and hence are not shown.

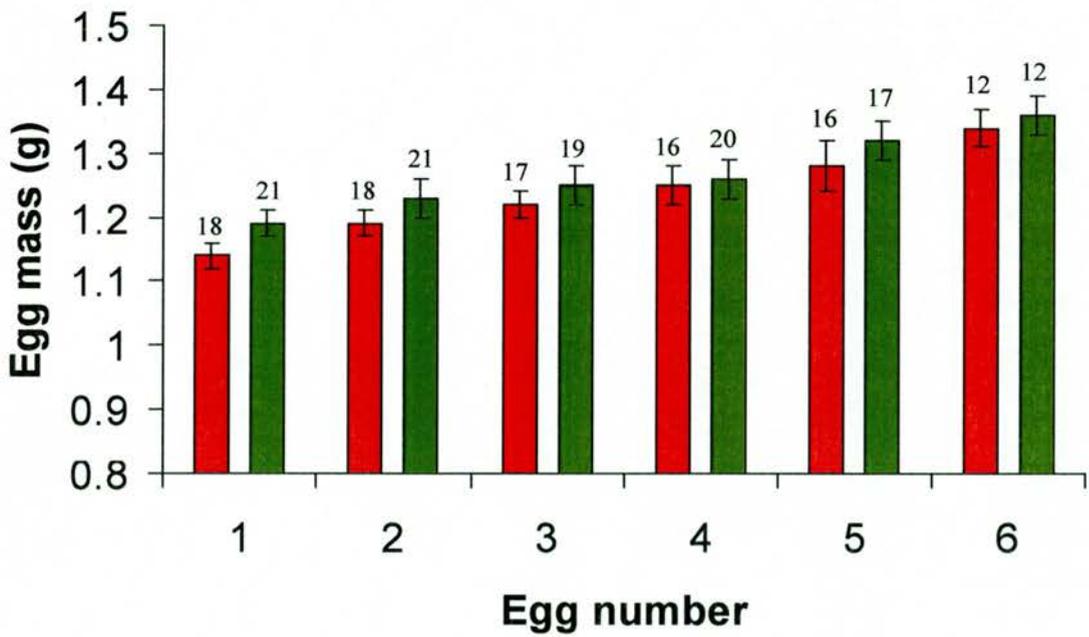
| <b>Variable</b>                                   | <b>df</b> | <b>F</b> | <b>p</b> |
|---|-----------|----------|----------|
| <b>Ring colour</b>                                | 1,34      | 0.21     | 0.65     |
| <b>Breeding round</b>                             | 1,34      | 6.52     | 0.02     |
| <b>Position in the laying sequence</b>            | 1,165     | 78.18    | < 0.0001 |
| <b>Female mass</b>                                | 1,37      | 18.91    | 0.0001   |
| <b>Ring colour* breeding round</b>                | 1,37      | 5.40     | 0.03     |
| <b>Female mass * breeding round</b>               | 1,34      | 6.88     | 0.01     |
| <b>Ring colour * female mass</b>                  | 1,34      | 0.26     | 0.61     |
| <b>Ring colour * female mass * breeding round</b> | 1,37      | 6.30     | 0.02     |

Figure 1 (overleaf). Mean egg mass ( $\pm$  SE) at each position in the laying sequence for females mated to red- and green-ringed males in (a) round one (b) round two and (c) round three. Females mated to red-ringed males are shown by red bars, females mated to green-ringed males are shown by green bars. Numbers of eggs are given above bars. Thirty-nine females laid eggs in both rounds one and two (with a different colour-ringed male in each round), 33 females also laid a clutch in round three (with a male of the same ring colour as in round two).

**a**



**b**



**c**

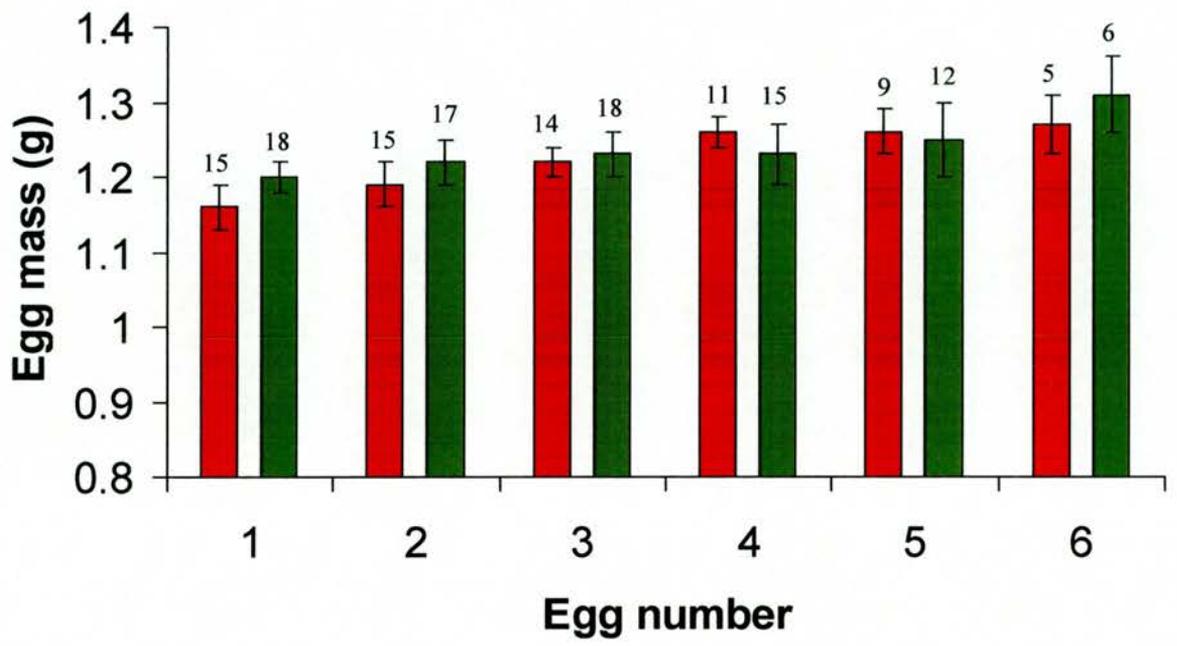
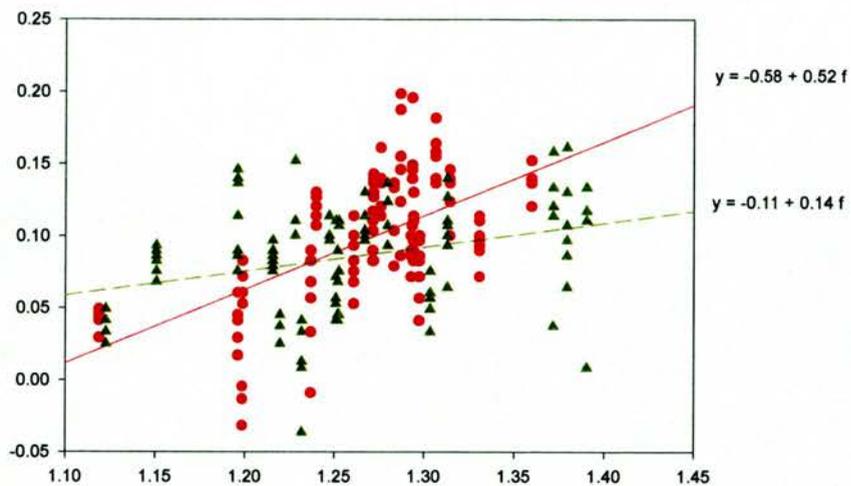
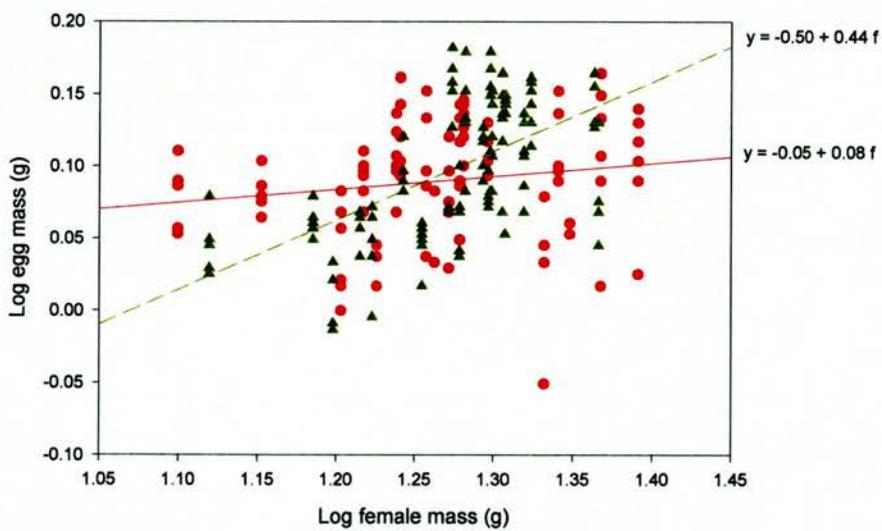


Figure 2 (overleaf). Allometric relationship between log egg mass and log female mass for females mated to red-and green-ringed males in (a) round one, (b) round two and (c) round three. The regression slopes are given in the form:  $y = a + b f$ , where  $y = \log$  egg mass,  $f = \log$  female mass. They are calculated from the GLM, which controls for the change in egg mass with laying sequence. Females mated to red-ringed males are shown by red circles and red, solid regression lines. Females mated to green-ringed males are shown by green triangles and green, dashed regression lines.

**a**



**b**



**c**

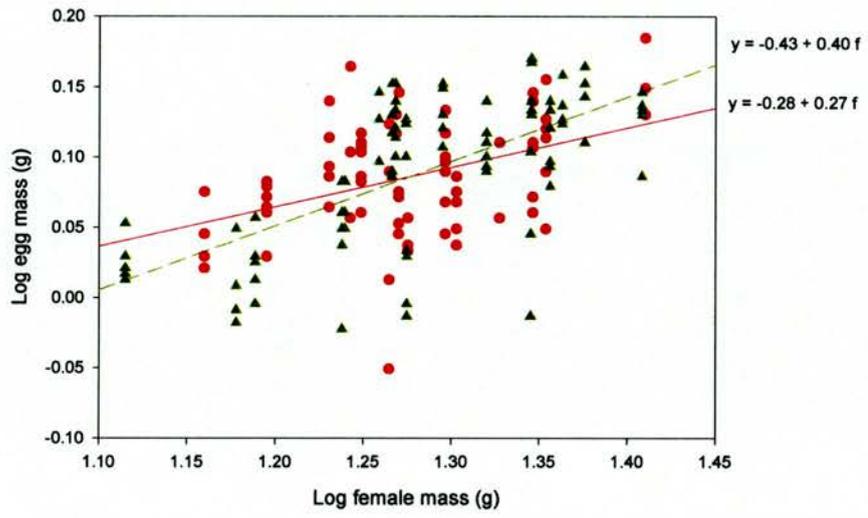


Table 3. Factors influencing the mass of eggs in rounds one and two (GLM analysis) for the 39 females that laid a clutch with both a red- and a green-ringed male. Non-significant terms were removed from the model and hence are not shown.

| <b>Variable</b>                        | <b>df</b> | <b>F</b> | <b>p</b> |
|--|-----------|----------|----------|
| <i>Round one</i>                       |           |          |          |
| <b>Ring colour</b>                     | 1,35      | 4.84     | 0.03     |
| <b>Position in the laying sequence</b> | 1,186     | 20.21    | < 0.0001 |
| <b>Female mass</b>                     | 1,35      | 20.21    | 0.0003   |
| <b>Ring colour * female mass</b>       | 1,35      | 5.60     | 0.02     |
| <i>Round two</i>                       |           |          |          |
| <b>Ring colour</b>                     | 1,34      | 6.88     | 0.01     |
| <b>Ring colour</b>                     | 1,36      | 4.05     | 0.052    |
| <b>Position in the laying sequence</b> | 1,175     | 34.30    | < 0.0001 |
| <b>Female mass</b>                     | 1,36      | 11.73    | 0.002    |
| <b>Clutch size</b>                     | 1,47      | 4.97     | 0.03     |
| <b>Ring colour * female mass</b>       | 1,36      | 4.95     | 0.03     |

In the second round of breeding, the relationship between female body mass, egg mass and ring colour was reversed. Females mated to green-ringed males tended to lay heavier eggs than females mated to red-ringed males (Fig. 1b) and again, in the final model (Table 3), there was a significant interaction between female body mass and ring colour (Fig. 2b). In this round, there was a significant positive relationship between egg mass and female body mass for females mated to green-ringed males ( $F_{1,20} = 16.15$ ,  $p = 0.0007$ ) but not for females mated to red-ringed males ( $F_{1,14} = 0.57$ ,  $p = 0.46$ ). Again, there was no difference in the mass of male and female eggs ( $F_{1,71} = 0.12$ ,  $p = 0.74$ ) and the interaction between ring colour and embryo sex was not significant ( $F_{1,70} = 1.01$ ,  $p = 0.32$ ).

This carry-over effect was evident in paired analyses for each order of treatment. For females that were paired with a red-ringed male in round one and a green-ringed male in round two, body mass explained a significant amount of variation in egg mass in both treatments ( $F_{1,41} = 31.03$ ,  $p < 0.0001$ ) but not for females that were paired with a green-ringed male in round one and a red-ringed male in round two ( $F_{1,13} = 2.09$ ,  $p = 0.17$ ). Ring colour had no effect in paired analyses for either order of treatment (red-ringed male first:  $F_{1,37} = 0.07$ ,  $p = 0.79$ , green-ringed male first:  $F_{1,20} = 0.01$ ,  $p = 0.91$ ).

In the third round of breeding, which was carried out to investigate this apparent carry-over effect, clutches were laid by 15/18 females mated to red-ringed males and 18/21 females mated to green-ringed males (all had laid clutches in both rounds one and two). There was no effect of ring colour on egg mass ( $F_{1,29} = 0.01$ ,  $p = 0.92$ , Fig. 1c). There was a significant positive relationship between body mass and egg mass both for females mated to red-ringed males ( $F_{1,16} = 8.66$ ,  $p = 0.01$ ) and for females mated to green-ringed males ( $F_{1,16} = 12.47$ ,  $p = 0.003$ , Fig. 2c). The interaction between female mass and ring colour with respect to egg mass was not significant ( $F_{1,29} = 0.52$ ,  $p = 0.48$ ).

### **Sex ratios and Mortality**

Second eggs were not included in the mortality analyses or in the analyses of the hatching and fledging sex ratios (see Methods). There was no difference in the proportion of male and female second eggs in either treatment in either round of breeding ( $p > 0.7$  in all cases). In round one, of the 17 removed, fertile second eggs in the red-ringed treatment, 9

were male and 8 were female. In the green-ringed treatment, 7 were male and 4 were female. In round two, in the red-ringed treatment 5 eggs were male and 6 were female, and in the green-ringed treatment, 6 eggs were male and 6 were female.

### Paired analyses

#### a) Sex ratio at laying

Twenty-four females laid fertile clutches when paired with both a red- and a green-ringed male (12 females were mated to a red-ringed male and 12 to a green-ringed male in round one). In a paired, generalized linear model analysis, there was no effect of ring colour on the mean clutch sex ratio (mean clutch sex ratio  $\pm$  SE, red-ringed pairs:  $0.42 \pm 0.06$ , green-ringed pairs:  $0.46 \pm 0.06$ ,  $F_{1,23} = 0.35$ ,  $p = 0.56$ , Fig. 3) and there was no effect of order of treatment (interaction between ring colour and breeding round:  $F_{1,22} = 0.79$ ,  $p = 0.38$ ). Six covariates of parental size and condition had no significant effects on the primary sex ratio and neither did clutch size (Table 4).

In addition, there was no relationship between sex and position in the laying sequence, i.e. male and female eggs were distributed randomly within the laying sequence ( $F_{1,145} = 0.00$ ,  $p = 0.99$ ) and there was no interaction between ring colour and laying sequence on embryo sex ( $F_{1,144} = 1.26$ ,  $p = 0.26$ ).

#### b) Sex ratio at hatching

Of these 24 females, two females did not hatch young with either male, four hatched young with just their red-ringed mate and four with just their green-ringed mate. Only 14 females hatched young in both treatments (six mated to a red-ringed male and eight to a green-ringed male in round one).

In a paired analysis of the sex ratio at hatching for these 14 females, there was a near significant effect of ring colour in the predicted direction, i.e. with a more female-biased brood sex ratio for green-ringed pairs (mean brood sex ratio  $\pm$  SE, red-ringed pairs:  $0.50 \pm 0.07$ , green-ringed pairs:  $0.35 \pm 0.08$ ,  $F_{1,13} = 4.22$ ,  $p = 0.058$ , Fig. 4b). There was no significant effect of order of treatment ( $F_{1,12} = 2.84$ ,  $p = 0.12$ ). When the sex ratio at laying was examined only for these fourteen females, there was not a significant difference between the mean primary clutch sex ratios (red-ringed pairs:  $0.52 \pm 0.06$ , green-ringed

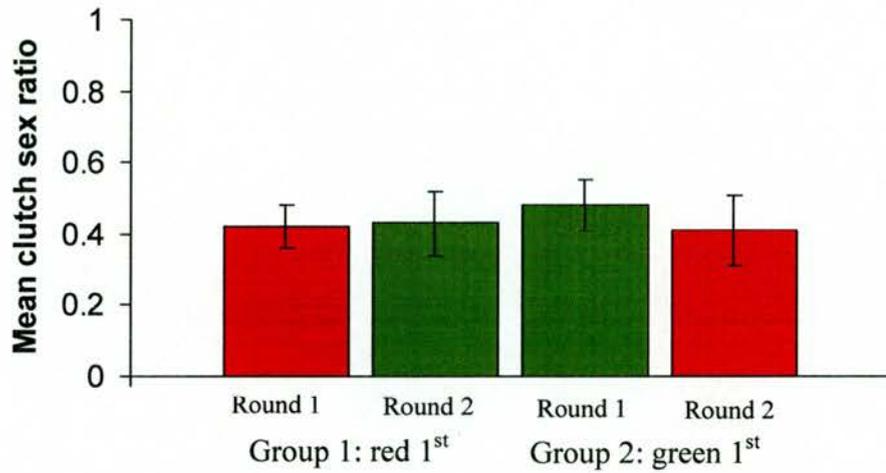


Figure 3. Paired sex ratio (proportion of males) at laying (means  $\pm$  SE) for all females that laid fertile clutches when mated to both a red-ringed male (represented by red bars) and a green-ringed male (represented by green bars). Group 1 females ( $n = 12$ ) were mated to a red-ringed male in round 1 and then to a green-ringed male in round 2. Group 2 females ( $n = 12$ ) were mated to a green-ringed male in round 1 and then to a red-ringed male in round 2.

Table 4. Results of a generalised linear model that tested the effects of male and female fat scores, male and female size (tarsus length), male and female condition (regression residual of body mass on tarsus length) and clutch size on the primary sex ratio. Data were paired for 24 females that laid fertile clutches with both a red- and a green-ringed male.

| <b>Variable</b>         | <b>df</b> | <b>F</b> | <b>p</b> |
|-------------------------|-----------|----------|----------|
| <b>Male condition</b>   | 1,17      | 0.00     | 0.98     |
| <b>Female condition</b> | 1,19      | 0.23     | 0.64     |
| <b>Clutch size</b>      | 1,20      | 0.58     | 0.45     |
| <b>Female tarsus</b>    | 1,21      | 0.96     | 0.34     |
| <b>Male fat score</b>   | 1,21      | 0.49     | 0.49     |
| <b>Female fat score</b> | 1,22      | 1.70     | 0.21     |
| <b>Male tarsus</b>      | 1,23      | 1.59     | 0.22     |

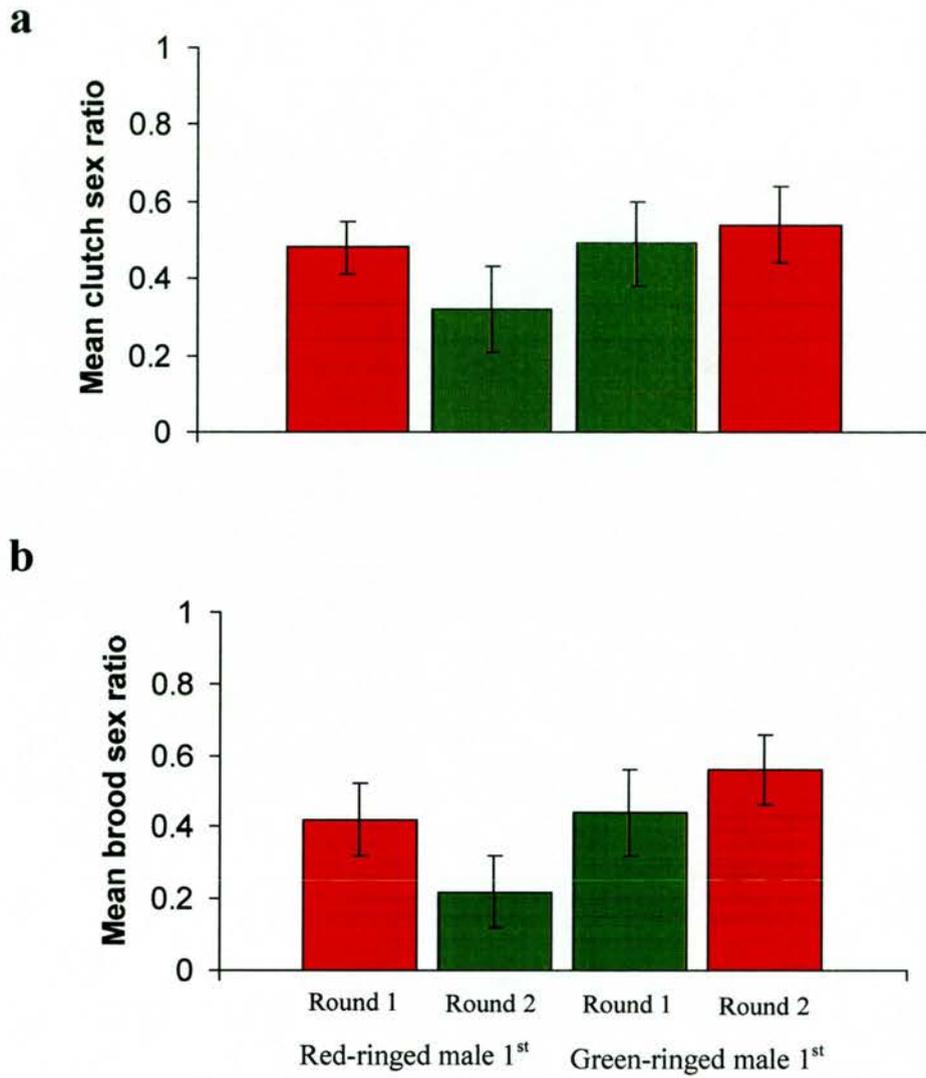


Figure 4. Mean brood sex ratio (proportion of males)  $\pm$  SE (a) at laying and (b) at hatching for females that laid a clutch and hatched young with both a red- and a green-ringed male. Six females bred with a red-ringed male and eight females bred with a green-ringed male in round one.

**c**

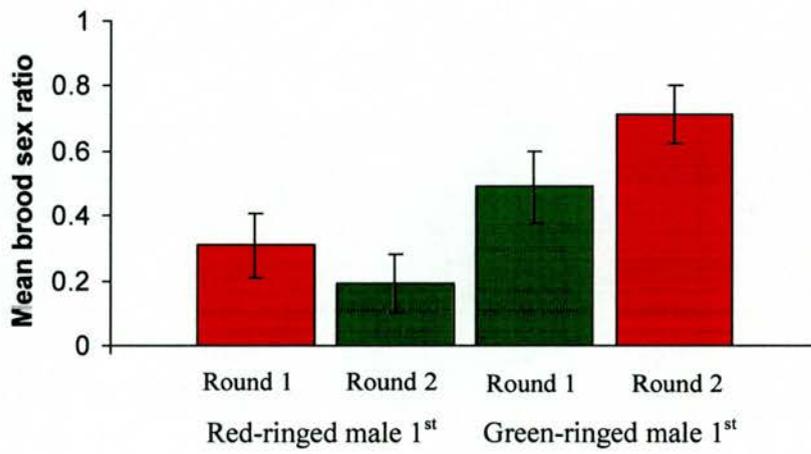


Figure 4c. Mean brood sex ratio ( $\pm$  SE) at fledging for females that fledged young with both a red- and a green-ringed male. Six females bred with a red-ringed male and seven females bred with a green-ringed male in round one.

pairs:  $0.42 \pm 0.08$ ,  $F_{1,13} = 2.29$ ,  $p = 0.15$ , Fig. 4a) and there was no significant order effect ( $F_{1,12} = 2.48$ ,  $p = 0.14$ ).

*c) Sex ratio at fledging*

Thirteen females fledged young in both treatments (six females mated to a red-ringed male and seven females mated to a green-ringed male in round one). There was a significant interaction between breeding round and ring colour ( $F_{1,11} = 7.76$ ,  $p = 0.02$ ), again indicating a shift in the sex ratio in the predicted direction (Fig. 4c). However, the change in sex ratio was not statistically significant either for females paired with a red-ringed male in round one and a green-ringed male in round two ( $F_{1,5} = 2.02$ ,  $p = 0.21$ ) or for females paired with a green-ringed male in round one and a red-ringed male in round two ( $F_{1,6} = 3.28$ ,  $p = 0.12$ ).

*d) Embryo mortality*

Paired data on embryo mortality were examined with respect to embryo sex, male ring colour, breeding round, position in the laying sequence and egg mass for 21 females that laid fertile clutches in both treatments (Table 5). Three females were excluded from the analysis since their only fertile egg was the second, removed, egg in one of their clutches. There was a significant effect of order of treatment ( $F_{1,112} = 4.67$ ,  $p = 0.03$ ). Females that were paired with red-ringed males in round one had relatively high embryo mortality both in this round (37.2% (16/43) of embryos died) and also in round two with their green-ringed mate (35.3% (12/34) of embryos died), whereas females that were paired with green-ringed males in round one had lower embryo mortality both in round one (17.8% (8/45)) and in round two (10% (4/40)). In round one, male embryo mortality (43.6% (17/39)) was greater than female embryo mortality (14.3% (7/49)),  $F_{1,66} = 9.50$ ,  $p = 0.003$ . However, the converse was true in round two (17.1% (6/35) of males compared to 25.6% (10/36) of females,  $F_{1,52} = 5.67$ ,  $p = 0.02$ ). Finally, there was a significant interaction between ring colour and position in the laying sequence ( $F_{1,112} = 7.77$ ,  $p = 0.006$ ). Embryo mortality tended to decrease with position in the laying sequence for red-ringed pairs ( $F_{1,57} = 3.82$ ,  $p = 0.056$ ), but there was no effect of laying sequence on embryo mortality for

Table 5. Results of multiple logistic regression analyses in which embryo mortality was examined in relation to ring colour, breeding round, embryo sex and position in the laying sequence. Paired data on embryo mortality were analysed for 21 females (162 embryos) that laid fertile clutches with both a red- and a green-ringed male. Non-significant terms, starting with the least significant interaction terms were removed from the model and hence not shown.

| <b>Variable</b>                      | <b>df</b> | <b>F</b> | <b>P</b> |
|--------------------------------------|-----------|----------|----------|
| <b>Ring colour</b>                   | 1,112     | 4.13     | 0.04     |
| <b>Breeding round</b>                | 1,38      | 0.06     | 0.81     |
| <b>Sex</b>                           | 1,112     | 2.44     | 0.12     |
| <b>Laying sequence</b>               | 1,112     | 2.61     | 0.11     |
| <b>Ring colour * breeding round</b>  | 1,112     | 4.67     | 0.03     |
| <b>Ring colour * laying sequence</b> | 1,112     | 7.77     | 0.006    |
| <b>Sex * breeding round</b>          | 1,112     | 13.29    | 0.0004   |

green-ringed pairs ( $F_{1,57} = 2.23$ ,  $p = 0.14$ ). Embryo mortality was not affected by egg mass in either treatment ( $F_{1,111} = 0.02$ ,  $p = 0.89$ ).

*e) Post-hatching mortality*

Post-hatching mortality was analysed in relation to treatment, chick sex, position in the laying sequence, breeding round, egg mass and brood size for the 14 females that hatched young in both treatments (Table 6). The single significant predictor of post-hatching mortality was egg mass. Chicks that hatched from heavier eggs had greater survival (Fig. 5). Importantly, given Burley's hypothesis that females may manipulate the sex ratio post-hatching depending on male attractiveness (Burley 1986b), the interaction between ring colour and sex was not significant ( $F_{1,62} = 1.62$ ,  $p = 0.21$ ).

**Separate analyses for each breeding round**

Due to the small sample size for females that hatched and fledged young in both rounds of breeding, unpaired data were also analysed for each breeding round separately. In addition, because egg mass data suggested that females were responding more to male ring colour in round one and not changing their resource allocation in round two, any effect on sex ratio between treatments should be evident in this analysis. The number of male and female chicks produced in each treatment in each round of breeding is summarized in Table 7. The number of deaths of male and female embryos and chicks in each breeding round is summarized in Table 8.

**Round one**

*a) Sex ratio at laying*

In round one, fertile clutches were laid by 17 females mated to red-ringed males and 15 females mated to green-ringed males. An equal proportion of chicks was produced in both treatments (Table 7a). The mean clutch sex ratio did not differ between treatments (mean clutch sex ratio  $\pm$  SE, red-ringed pairs:  $0.43 \pm 0.05$ , green-ringed pairs:  $0.50 \pm 0.06$ ,  $F_{1,30} = 0.42$ ,  $p = 0.52$ ). There was no relationship between sex and position in the laying sequence ( $F_{1,117} = 0.14$ ,  $p = 0.71$ ) and no interaction between ring colour and laying sequence on embryo sex ( $F_{1,116} = 0.24$ ,  $p = 0.63$ ).

Table 6. Results of multiple logistic regression analyses in which post-hatching mortality was examined in relation to ring colour, breeding round, sex, position in the laying sequence, brood size and egg mass. Paired data were analysed for 14 females that hatched young in both treatments (110 chicks).

| <b>Post-hatching mortality</b> | <b>df</b> | <b>F</b> | <b>p</b> |
|--------------------------------|-----------|----------|----------|
| <b>Sex</b>                     | 1,61      | 0.22     | 0.64     |
| <b>Breeding round</b>          | 1,24      | 0.33     | 0.57     |
| <b>Ring colour</b>             | 1,62      | 0.21     | 0.65     |
| <b>Laying sequence</b>         | 1,63      | 0.81     | 0.37     |
| <b>Brood size</b>              | 1,64      | 0.49     | 0.49     |
| <b>Egg mass</b>                | 1,65      | 8.82     | 0.004    |

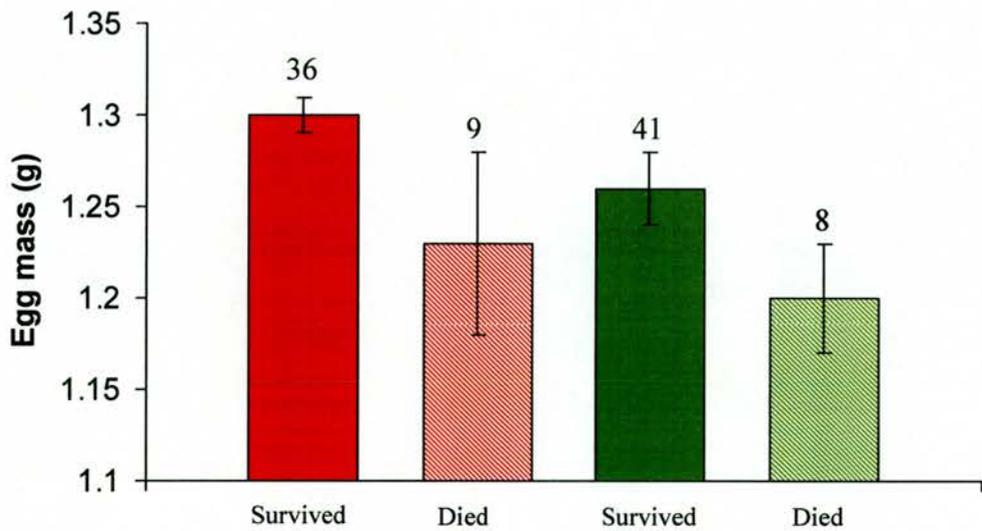


Figure 5. Mass of eggs (mean  $\pm$  SE) from which chicks hatched, that survived to fledging (filled bars) or died before fledging (hatched bars). Red bars represent eggs laid by females mated to red-ringed males. Green bars represent eggs laid by females mated to green-ringed males. Data are pooled for females that hatched young in both rounds of breeding ( $n = 14$ ). Number of eggs are given above bars.

Table 7. The number of chicks of each sex at laying, hatching and fledging for females that were mated with a red-ringed and a green-ringed male in (a) round one and (b) round two. All females in round two had also laid clutches in round one. Sex ratio = males / (males + females).  $G_{adj 1}$  values are goodness of fit against the Mendelian expectation of equal numbers of both sexes (\*  $p < 0.05$ , \*\*  $p < 0.01$ ). M = males, F = females.

|                   | Laying        |       |           | Hatching    |               |       | Fledging  |             |               |       |           |             |
|-------------------|---------------|-------|-----------|-------------|---------------|-------|-----------|-------------|---------------|-------|-----------|-------------|
|                   | No. of broods | M/F   | Sex ratio | $G_{adj 1}$ | No. of broods | M/F   | Sex ratio | $G_{adj 1}$ | No. of broods | M/F   | Sex ratio | $G_{adj 1}$ |
| <b>a</b>          |               |       |           |             |               |       |           |             |               |       |           |             |
| Red-ringed mate   | 17            | 37/44 | 0.46      | 0.60        | 14            | 14/28 | 0.33      | 4.70 *      | 11            | 6/23  | 0.21      | 10.45 **    |
| Green-ringed mate | 15            | 35/35 | 0.50      | 0.00        | 13            | 20/24 | 0.45      | 0.36        | 11            | 18/20 | 0.47      | 0.10        |
| <b>b</b>          |               |       |           |             |               |       |           |             |               |       |           |             |
| Red-ringed mate   | 15            | 30/27 | 0.53      | 0.16        | 12            | 26/17 | 0.60      | 1.88        | 11            | 24/14 | 0.63      | 2.63        |
| Green-ringed mate | 17            | 28/36 | 0.44      | 0.99        | 13            | 16/26 | 0.38      | 2.38        | 13            | 12/23 | 0.34      | 3.47        |

Table 8. Number of male and female deaths, pre- and post-hatching, in each ring colour treatment in the first and second rounds of breeding. In round one 17 females were mated to red-ringed males and 15 to green-ringed males. In round two 15 females were mated to red-ringed males and 17 to green-ringed males. All females in round two had also laid clutches in round one.

|                     | Embryo mortality |         | Post-hatching mortality |         |
|---------------------|------------------|---------|-------------------------|---------|
|                     | Males            | Females | Males                   | Females |
| <i>Round one</i>    |                  |         |                         |         |
| <b>Red-ringed</b>   | 17/27            | 7/39    | 4/10                    | 11/32   |
| <b>Green-ringed</b> | 9/29             | 6/30    | 2/20                    | 4/24    |
| <i>Round two</i>    |                  |         |                         |         |
| <b>Red-ringed</b>   | 0/26             | 4/21    | 2/26                    | 3/17    |
| <b>Green-ringed</b> | 9/23             | 7/31    | 5/14                    | 2/24    |

*b) Sex ratio at hatching*

Twenty-seven pairs hatched young in round one (14 red-ringed pairs and 13 green-ringed pairs). More female chicks hatched in the red-ringed treatment whereas an equal proportion of males and females hatched in the green-ringed treatment (Table 7a). However, the mean brood sex ratio was not significantly different between the two treatments (mean brood sex ratio  $\pm$  SE, red-ringed pairs:  $0.31 \pm 0.08$ , green-ringed pairs:  $0.50 \pm 0.08$ ,  $F_{1,25} = 3.11$ ,  $p = 0.09$ ).

*c) Sex ratio at fledging*

Twenty-two pairs fledged young in round one (11 in each treatment). Again, more female chicks fledged in the red-ringed treatment whereas an equal proportion of males and females hatched in the green-ringed treatment (Table 7a). There was a significant difference in the mean brood sex ratio between treatments (mean brood sex ratio  $\pm$  SE, red-ringed pairs:  $0.19 \pm 0.06$ , green-ringed pairs:  $0.54 \pm 0.08$ ,  $F_{1,20} = 7.75$ ,  $p = 0.01$ ).

*d) Embryo mortality*

Male embryo mortality was significantly higher than female embryo mortality ( $F_{1,92} = 8.35$ ,  $p = 0.005$ ). This pattern was greater, although not significantly so, among red-ringed pairs compared with green-ringed pairs (red-ringed pairs: 63% (17/27) of males compared to 17.9% (7/39) of females,  $F_{1,47} = 8.46$ ,  $p = 0.006$ , green-ringed pairs: 31% (9/29) of males compared to 20% (6/30) of females,  $F_{1,42} = 0.26$ ,  $p = 0.61$ , interaction between ring colour and sex:  $F_{1,91} = 2.70$ ,  $p = 0.10$ ). There was no effect of laying sequence on embryo mortality ( $F_{1,90} = 0.53$ ,  $p = 0.47$ ). Egg mass had a significant effect on embryo mortality of eggs laid by females mated to red-ringed males. Unexpectedly, embryo mortality was higher for heavier eggs ( $F_{1,47} = 4.91$ ,  $p = 0.03$ , Fig. 6a), but there was no effect of egg mass on embryo mortality for green-ringed pairs ( $F_{1,43} = 1.16$ ,  $p = 0.29$ , Fig. 6a). The interaction between egg mass and ring colour was not statistically significant ( $F_{1,90} = 2.82$ ,  $p = 0.094$ ).

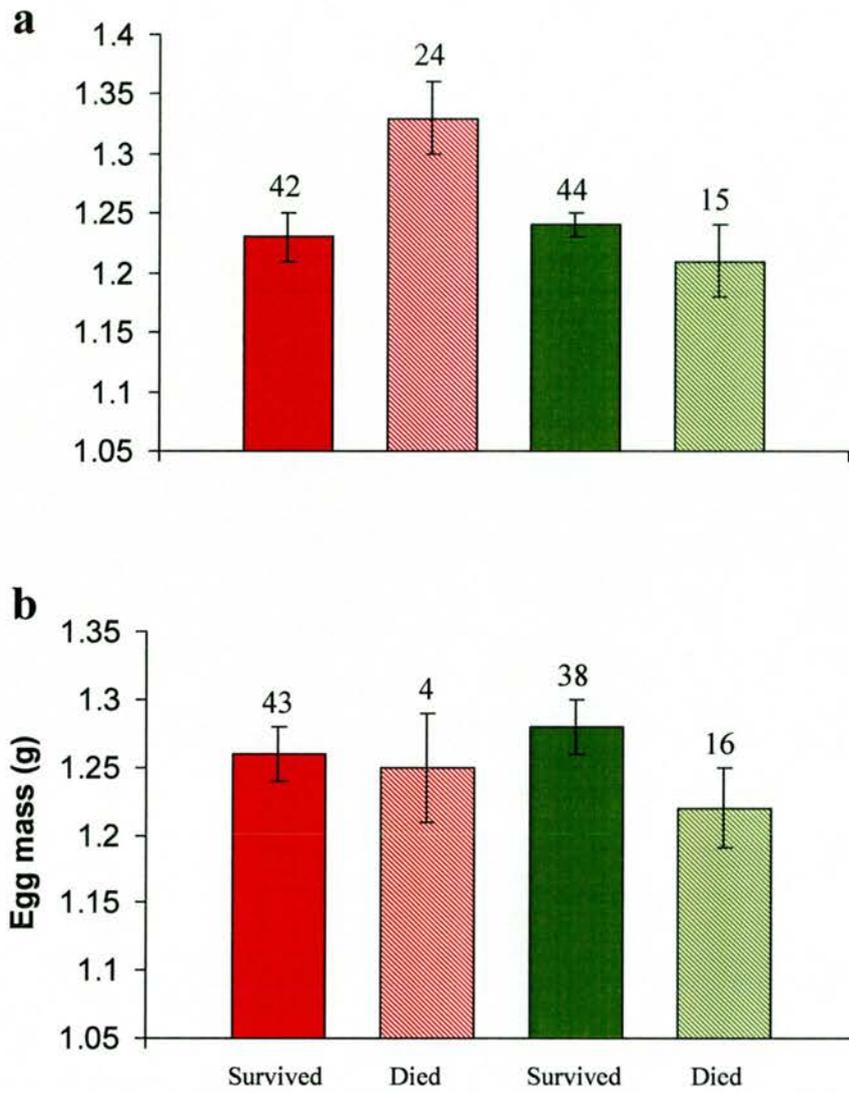


Figure 6. Mass (mean  $\pm$  SE) of successful eggs from which embryos survived to hatching (filled bars) and unsuccessful eggs in which embryos died (hatched bars) in (a) round one and (b) round two of breeding. Red bars represent eggs laid by females mated to red-ringed males. Green bars represent eggs laid by females mated to green-ringed males. Numbers of eggs are given above bars.

*e) Post-hatching mortality*

In round one, the nestling mortality between hatching and fledging tended to be higher among offspring of red-ringed pairs (35.7% (15/42)) than that of green-ringed pairs (13.6% (6/44), Tables 8 & 9). Egg mass was a significant predictor of post-hatch mortality with chicks from heavier eggs having significantly higher survivorship than chicks from lighter eggs ( $F_{1,58} = 11.08$ ,  $p = 0.002$ ). Importantly, again, given Burley's hypothesis that sex ratio manipulation may occur post-hatching (Burley 1986b), there was no sex-biased mortality ( $F_{1,56} = 0.76$ ,  $p = 0.39$ ) and no interaction between ring colour and sex on post-hatching mortality ( $F_{1,55} = 0.28$ ,  $p = 0.60$ ).

**Round two**

*a) Sex ratio at laying*

In round two fertile clutches were laid by 15 females mated to red-ringed males and 17 females mated to green-ringed males. As in round one, an equal proportion of male and female eggs was produced in both treatments (Table 7b), and the mean clutch sex ratio did not differ between treatments (mean clutch sex ratio  $\pm$  SE, red-ringed pairs:  $0.41 \pm 0.09$ , green-ringed pairs:  $0.47 \pm 0.08$ ,  $F_{1,30} = 0.76$ ,  $p = 0.39$ ). Again, there was no relationship between sex and position in the laying sequence ( $F_{1,79} = 0.32$ ,  $p = 0.57$ ) and no interaction between ring colour and laying sequence on embryo sex ( $F_{1,78} = 2.11$ ,  $p = 0.15$ ).

*b) Sex ratio at hatching*

Twenty-five pairs hatched young in round two (12 red-ringed pairs and 13 green-ringed pairs). In this round there was a non-significant tendency for more female chicks to hatch in the green-ringed treatment (Table 7b). The difference in the mean brood sex ratio between treatments approached significance (mean brood sex ratio  $\pm$  SE, red-ringed pairs:  $0.54 \pm 0.08$ , green-ringed pairs:  $0.34 \pm 0.09$ ,  $F_{1,23} = 4.00$ ,  $p = 0.058$ ).

*c) Sex ratio at fledging*

Twenty-four pairs fledged young in round two (11 red-ringed pairs and 13 green-ringed pairs). There was a non-significant tendency for more males to fledge in the red-ringed treatment and for more females to fledge in the green-ringed treatment (Table 7b). There

Table 9. Results of multiple logistic regression analyses in which post-hatching mortality was examined in relation to ring colour, sex, position in the laying sequence, brood size and egg mass in rounds one and two.

| <b>Post-hatching mortality</b> | <b>df</b> | <b>F</b> | <b>p</b> |
|--------------------------------|-----------|----------|----------|
| <i>Round 1</i>                 |           |          |          |
| <b>Sex</b>                     | 1,56      | 0.18     | 0.68     |
| <b>Laying sequence</b>         | 1,57      | 2.32     | 0.13     |
| <b>Brood size</b>              | 1,58      | 3.04     | 0.09     |
| <b>Ring colour</b>             | 1,58      | 3.64     | 0.06     |
| <b>Egg mass</b>                | 1,58      | 10.56    | 0.002    |
| <i>Round 2</i>                 |           |          |          |
| <b>Brood size</b>              | 1,54      | 0.24     | 0.63     |
| <b>Laying sequence</b>         | 1,54      | 2.30     | 0.14     |
| <b>Egg mass</b>                | 1,55      | 3.51     | 0.07     |
| <b>Ring colour</b>             | 1,55      | 0.83     | 0.37     |
| <b>Sex</b>                     | 1,55      | 5.92     | 0.02     |
| <b>Ring colour * sex</b>       | 1,55      | 7.32     | 0.009    |

was a significant difference in the mean brood sex ratios between treatments (mean brood sex ratio  $\pm$  SE, red-ringed pairs:  $0.63 \pm 0.08$ , green-ringed pairs:  $0.31 \pm 0.09$ ,  $F_{1,22} = 6.16$ ,  $p = 0.02$ ).

*d) Embryo mortality*

Embryo mortality was significantly affected by egg mass, with successful eggs being significantly heavier than unsuccessful eggs ( $F_{1,70} = 4.60$ ,  $p = 0.04$ , Fig. 6b). Male embryo mortality was not significantly different from that of female embryo mortality ( $F_{1,69} = 2.31$ ,  $p = 0.13$ , Table 8), and although the embryo mortality tended to be higher among green-ringed pairs (Table 8), this was not statistically significant ( $F_{1,69} = 2.62$ ,  $p = 0.11$ ). There was no effect of position in the laying sequence on embryo mortality ( $F_{1,68} = 2.04$ ,  $p = 0.16$ ). The interaction between ring colour and embryo sex on embryo mortality was not significant ( $F_{1,67} = 0.00$ ,  $p = 0.99$ ).

*e) Post-hatching mortality*

In round two (Tables 8 & 9), there was a significant interaction between ring colour and chick sex ( $F_{1,55} = 7.32$ ,  $p = 0.009$ ). For red-ringed pairs chick mortality was low for both males (8.3% (2/24) of chicks) and females (17.6% (3/17) of chicks), effect of sex:  $F_{1,30} = 0.37$ ,  $p = 0.55$ ). For green-ringed pairs, male chick mortality tended to be greater than that of females (55.6% (5/9) of males compared to 8.3% (2/24) of females,  $F_{1,26} = 3.62$ ,  $p = 0.07$ ).

**Androgen analysis**

Paired analyses were carried out on the androgen levels of second eggs from 32 females (assay data were unavailable for seven females). There was no effect of yolk mass on T concentration ( $F_{1,26} = 0.01$ ,  $p = 0.92$ ) or DHT concentration ( $F_{1,26} = 0.15$ ,  $p = 0.70$ ). T concentration was significantly higher in round one than in round two ( $F_{1,31} = 9.14$ ,  $p = 0.005$ ) but there was no difference between rounds one and two for DHT concentration ( $F_{1,30} = 0.25$ ,  $p = 0.62$ ). There was no effect of ring colour on T concentration ( $F_{1,29} = 0.53$ ,  $p = 0.47$ ) or on DHT concentration ( $F_{1,30} = 0.25$ ,  $p = 0.62$ ) and there was no effect of order of treatment (interaction between breeding round and ring colour, T:  $F_{1,27} = 0.18$ ,  $p = 0.68$ ,

DHT:  $F_{1,27} = 1.29$ ,  $p = 0.27$ ). Finally, male and female eggs did not differ with respect to T ( $F_{1,32} = 0.00$ ,  $p = 0.99$ ) or DHT concentration ( $F_{1,32} = 0.54$ ,  $p = 0.47$ ) and there was no interaction between ring colour and embryo sex for either T ( $F_{1,31} = 0.03$ ,  $p = 0.87$ ) or DHT ( $F_{1,26} = 0.47$ ,  $p = 0.50$ ).

Clutch size and two covariates of female condition (fat score and the regression residual of body mass on tarsus length) had no effects on T concentration (Table 10). However, there was a significant effect of fat score on DHT concentration ( $F_{1,37} = 4.98$ ,  $p = 0.03$ ): females with lower fat scores laid eggs with higher DHT concentrations (Fig. 7).

### Offspring development

#### *Paired analyses:*

Paired data for hatchlings were analysed from 14 pairs (six females were mated with a red-ringed male and eight females were mated with a green-ringed male in round one, Table 11) and paired data for fledglings and adults were analysed from 13 pairs (one of the females that bred with a green-ringed male in round one fledged no chicks). There was no difference in the number of young fledged between treatments (mean number of fledglings  $\pm$  SE, red-ringed pairs:  $3.08 \pm 0.33$ , green-ringed pairs:  $3.08 \pm 0.35$ , paired t-test,  $t = 0.00$ ,  $p = 1.00$ ).

Hatching mass, fledging mass and adult mass were all strongly related to egg mass (Table 11). Ring colour and chick sex had no significant effects on any measurements of offspring development, and all interactions between ring colour and sex were non significant ( $p > 0.1$  in all cases). Laying sequence had a negative effect on age at fledging. There was no effect of order of treatment with respect to any of the measurements ( $p > 0.1$  in all cases).

#### *Separate analyses for each breeding round*

Data were analysed for 14 red and 13 green broods that hatched, and for 11 red and 11 green broods that fledged in the first round of breeding. There was no difference in the number of offspring that fledged between treatments (mean number of fledglings  $\pm$  SE, red-ringed pairs:  $2.64 \pm 0.34$ , green-ringed pairs:  $3.45 \pm 0.39$ , independent samples t-test,  $t = 1.59$ ,  $df = 20$ ,  $p = 0.13$ ).

Table 10. Results of a General Linear Model that tested the effects of female fat scores, female condition (regression residual of body mass on tarsus length) and clutch size on yolk androgen concentrations (T and DHT). Data were paired for 32 females that laid clutches with both a red- and a green-ringed male.

| <b>Variable</b>                   | <b>df</b> | <b>F</b> | <b>P</b> |
|-----------------------------------|-----------|----------|----------|
| <i>T concentration</i>            |           |          |          |
| <b>Residual of tarsus on mass</b> | 1,32      | 0.14     | 0.71     |
| <b>Fat score</b>                  | 1,37      | 0.56     | 0.46     |
| <b>Clutch size</b>                | 1,57      | 2.82     | 0.10     |
| <i>DHT concentration</i>          |           |          |          |
| <b>Clutch size</b>                | 1,55      | 1.32     | 0.26     |
| <b>Residual of tarsus on mass</b> | 1,35      | 2.01     | 0.17     |
| <b>Fat score</b>                  | 1,37      | 4.98     | 0.03     |

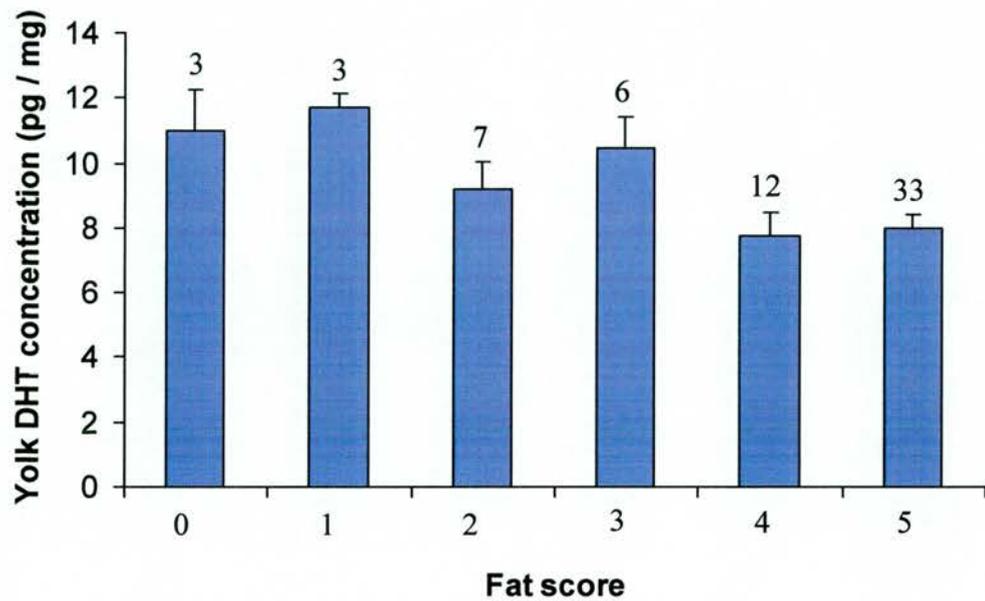


Figure 7. Yolk DHT concentration (means  $\pm$  SE) in relation to female fat scores. Data are pooled for females that laid clutches with both a red- and a green-ringed male ( $n = 32$ ). Numbers of eggs that were assayed are given above bars.

Table 11. Paired analyses of offspring development for females that bred with both a red- and a green-ringed male. Non-significant terms were sequentially removed from the model, starting with the least significant interaction term.

| Trait              | Colour |      | Laying sequence |      | Sex   |          | Egg mass |      |      |      |       |          |
|--------------------|--------|------|-----------------|------|-------|----------|----------|------|------|------|-------|----------|
|                    | df     | F    | p               | df   | F     | p        | df       | F    | p    |      |       |          |
| Hatchling mass (g) | 1,46   | 3.03 | 0.09            | 1,72 | 1.38  | 0.24     | 1,79     | 1.59 | 0.21 | 1,62 | 13.13 | 0.0006** |
| Hatch tarsus (mm)  | 1,28   | 1.19 | 0.28            | 1,77 | 0.16  | 0.69     | 1,66     | 0.94 | 0.34 | 1,70 | 0.02  | 0.89     |
| Days to fledging   | 1,22   | 0.61 | 0.44            | 1,71 | 12.96 | 0.0006** | 1,57     | 1.26 | 0.27 | 1,54 | 0.67  | 0.42     |
| Fledgling mass (g) | 1,16   | 0.22 | 0.64            | 1,61 | 2.15  | 0.15     | 1,59     | 1.14 | 0.29 | 1,45 | 11.06 | 0.002**  |
| Fledge tarsus (mm) | 1,39   | 1.71 | 0.20            | 1,66 | 0.06  | 0.81     | 1,65     | 0.95 | 0.33 | 1,53 | 4.72  | 0.03     |
| Adult mass (g)     | 1,26   | 1.55 | 0.22            | 1,61 | <0.01 | 0.94     | 1,59     | 0.02 | 0.90 | 1,37 | 10.14 | 0.003**  |
| Adult tarsus (mm)  | 1,29   | 0.06 | 0.81            | 1,66 | 0.27  | 0.60     | 1,60     | 2.64 | 0.11 | 1,45 | 4.58  | 0.04     |

Data were analysed for 12 red and 13 green broods hatched in the second round of breeding, and for 11 red and 13 green broods fledged in the second round of breeding. Again, there was no difference in the number of offspring fledged in each treatment (mean number of fledglings  $\pm$  SE, red-ringed pairs:  $3.73 \pm 0.27$ , green-ringed pairs:  $3.08 \pm 0.33$ , independent samples t-test,  $t = 1.49$ ,  $df = 22$ ,  $p = 0.15$ ).

Ring colour had no significant effects on offspring development in either round (Tables 12a & 12b). Again, there was no effect of chick sex in either round and no significant interactions between sex and ring colour ( $p > 0.05$  in all cases).

### **Egg macro-composition (round three)**

Both wet and dry, albumen and yolk mass were positively correlated with egg mass in both colour treatments such that heavier eggs contained greater absolute quantities of all egg components (Table 13).

There was a significant interaction between log egg mass and ring colour with respect to log wet yolk mass ( $F_{1,89} = 4.30$ ,  $p = 0.04$ , Fig. 8a) and log dry yolk mass ( $F_{1,85} = 4.40$ ,  $p = 0.04$ , Fig. 8b). In the analysis for log wet albumen mass there was no significant effect of ring colour ( $F_{1,32} = 0.05$ ,  $p = 0.82$ ) and no significant interaction between ring colour and log egg mass ( $F_{1,88} = 0.79$ ,  $p = 0.38$ ). However, there was a significant effect of laying sequence ( $F_{1,95} = 4.90$ ,  $p = 0.03$ ) with later laid eggs containing proportionately more wet albumen. The same was true in the analysis of log dry albumen. There was no significant effect of ring colour ( $F_{1,37} = 0.11$ ,  $p = 0.75$ ) and no significant interaction between ring colour and log egg mass ( $F_{1,91} = 2.66$ ,  $p = 0.11$ ). However, again, there was a significant effect of laying sequence ( $F_{1,97} = 5.39$ ,  $p = 0.02$ ) with later laid eggs containing proportionately more dry albumen.

There was no difference in the carotenoid content as indicated by the Roche colour scores between eggs of red- and green-ringed pairs ( $F_{1,29} = 0.80$ ,  $p = 0.38$ ) but there was a significant decrease in the yolk colour score in both treatments with laying sequence ( $F_{1,82} = 22.52$ ,  $p < 0.0001$ ).

Table 12a. Factors affecting development of offspring in round one of breeding. N = 14 red pairs and 13 green pairs. Non-significant terms were sequentially removed from the model, starting with the least significant interaction term.

| Trait                | Ring colour |      |      | Laying order |      |       | Sex  |      |      | Egg mass |       |           |
|----------------------|-------------|------|------|--------------|------|-------|------|------|------|----------|-------|-----------|
|                      | df          | F    | p    | df           | F    | p     | df   | F    | p    | df       | F     | p         |
| Hatching mass (g)    | 1,26        | 3.42 | 0.08 | 1,73         | 3.56 | 0.06  | 1,61 | 2.62 | 0.11 | 1,47     | 18.12 | <0.0001** |
| Hatch tarsus (mm)    | 1,27        | 0.77 | 0.39 | 1,79         | 0.10 | 0.75  | 1,56 | 0.09 | 0.76 | 1,72     | 0.03  | 0.87      |
| Days to fledging     | 1,20        | 2.23 | 0.15 | 1,65         | 6.14 | 0.02* | 1,49 | 3.69 | 0.06 | 1,41     | 0.46  | 0.50      |
| Fledging mass (g)    | 1,25        | 2.82 | 0.11 | 1,60         | 2.16 | 0.15  | 1,50 | 2.07 | 0.16 | 1,44     | 19.42 | <0.0001** |
| Fledging tarsus (mm) | 1,29        | 0.02 | 0.89 | 1,61         | 0.25 | 0.62  | 1,59 | 0.08 | 0.78 | 1,42     | 5.09  | 0.03*     |
| Adult mass (g)       | 1,25        | 0.00 | 0.95 | 1,59         | 0.21 | 0.65  | 1,53 | 0.31 | 0.58 | 1,40     | 2.31  | 0.14      |
| Adult tarsus (mm)    | 1,31        | 0.02 | 0.90 | 1,59         | 0.03 | 0.85  | 1,58 | 0.31 | 0.58 | 1,43     | 0.31  | 0.58      |

Table 12b. Factors affecting development of offspring in round two of breeding. N = 12 red pairs and 12 green pairs. Non-significant terms were sequentially removed from the model, starting with the least significant interaction term.

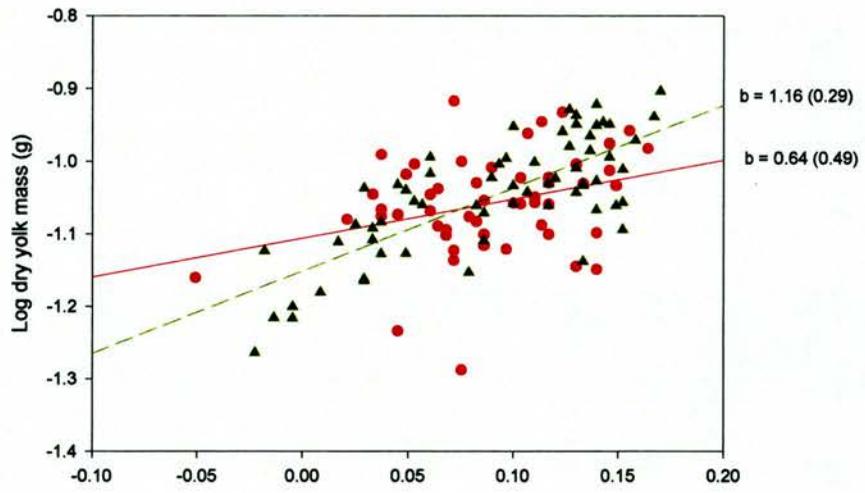
| Trait                       | Ring colour |      | Laying order |      | Sex  |      | Egg mass |      |      |       |           |
|-----------------------------|-------------|------|--------------|------|------|------|----------|------|------|-------|-----------|
|                             | df          | F    | p            | df   | F    | p    | df       | F    | p    |       |           |
| <b>Hatching mass (g)</b>    | 1,33        | 1.17 | 0.29         | 1,66 | 0.02 | 0.90 | 1,77     | 2.35 | 0.13 | 56.08 | <0.0001** |
| <b>Hatch tarsus (mm)</b>    | 1,25        | 0.02 | 0.88         | 1,54 | 0.18 | 0.67 | 1,69     | 3.61 | 0.06 | 8.25  | 0.006**   |
| <b>Days to fledging</b>     | 1,18        | 3.54 | 0.08         | 1,66 | 1.99 | 0.16 | 1,45     | 0.49 | 0.49 | 6.31  | 0.01*     |
| <b>Fledging mass (g)</b>    | 1,16        | 0.14 | 0.71         | 1,67 | 1.45 | 0.23 | 1,42     | 0.12 | 0.73 | 0.55  | 0.46      |
| <b>Fledging tarsus (mm)</b> | 1,29        | 0.01 | 0.93         | 1,64 | 0.20 | 0.66 | 1,65     | 0.95 | 0.33 | 3.45  | 0.07      |
| <b>Adult mass (g)</b>       | 1,19        | 0.46 | 0.51         | 1,64 | 1.31 | 0.26 | 1,49     | 2.36 | 0.13 | 4.25  | 0.045*    |
| <b>Adult tarsus (mm)</b>    | 1,21        | 0.45 | 0.51         | 1,62 | 0.01 | 0.92 | 1,49     | 0.61 | 0.44 | 2.31  | 0.14      |

Table 13. Slopes  $\pm$  95% confidence intervals (C.I.) of regressions for log egg component on log egg mass for eggs laid by red- and green-mated females in round three. Asterisks indicate slopes that differ significantly from 1.0.

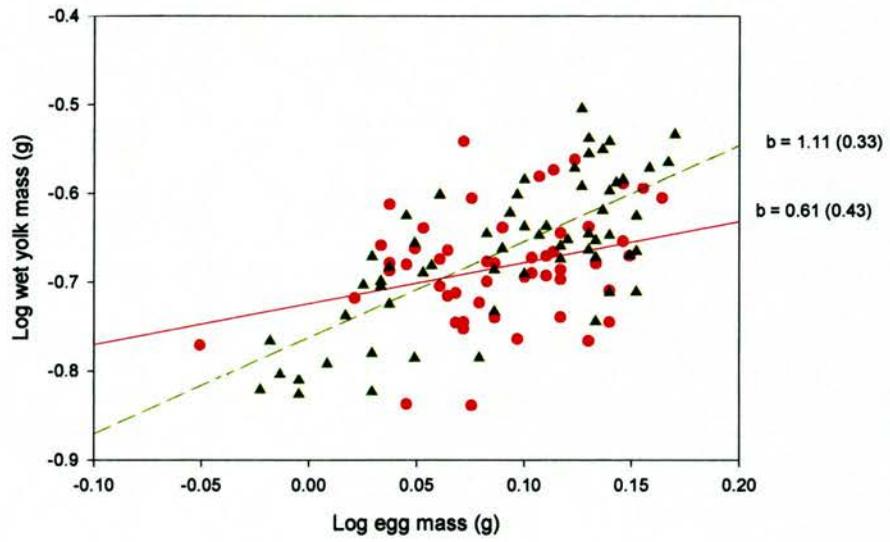
|                                       | <b>Egg component</b> | <b>Slope <math>\pm</math><br/>95% C.I.</b> | <b>F</b>                  | <b>p</b> |
|---------------------------------------|----------------------|--|---------------------------|----------|
| <b><i>Red-mated<br/>females</i></b>   | Log wet yolk mass    | 0.61 $\pm$ 0.43                            | F <sub>1,50</sub> = 8.38  | 0.006    |
|                                       | Log dry yolk mass    | 0.64 $\pm$ 0.49                            | F <sub>1,49</sub> = 7.07  | 0.01     |
|                                       | Log wet albumen mass | 1.58 $\pm$ 0.45 *                          | F <sub>1,48</sub> = 49.41 | < 0.0001 |
|                                       | Log dry albumen mass | 1.77 $\pm$ 0.45 *                          | F <sub>1,47</sub> = 62.26 | < 0.0001 |
| <b><i>Green-mated<br/>females</i></b> | Log wet yolk mass    | 1.11 $\pm$ 0.33                            | F <sub>1,28</sub> = 49.05 | < 0.0001 |
|                                       | Log dry yolk mass    | 1.16 $\pm$ 0.29                            | F <sub>1,28</sub> = 68.65 | < 0.0001 |
|                                       | Log wet albumen mass | 1.21 $\pm$ 0.33                            | F <sub>1,32</sub> = 55.23 | < 0.0001 |
|                                       | Log dry albumen mass | 1.10 $\pm$ 0.41                            | F <sub>1,35</sub> = 29.42 | < 0.0001 |

Figure 8. Allometric regression plots of a) log wet yolk mass and b) log dry yolk mass in relation to log egg mass for red- (n = 15) and green-mated females (n = 18) in round three. Slopes (95% confidence intervals) are given. Females mated to red-ringed males are shown by red circles and red, solid regression lines. Females mated to green-ringed males are shown by green triangles and green, dashed regression lines.

**a**



**b**



## Discussion

### Evidence for differential allocation

This study found evidence for differential allocation at laying by females in relation to the attractiveness of their mates, in the absence of assortative mating. Females mated to attractive males did not start laying earlier or lay larger clutches but, in the first round of breeding, they laid heavier eggs and invested more relative to their body mass compared to females mated to unattractive males. The androgen analyses in the current study did not suggest that females mated to attractive males were depositing greater concentrations of T and DHT as found by Gil *et al.* (1999). However, these authors assayed fresh eggs, while the current study used eggs that had been incubated for 96 hours to obtain information on sex allocation. Embryonic development can significantly affect the levels of yolk androgens (Elf & Fivizzani 2002) and the data may not, therefore, represent initial deposition by females.

### Carry-over effect

It might be expected that females that invested more heavily in one round of breeding would invest less heavily in a subsequent breeding round and vice-versa. However, in the second round, the females maintained the same pattern of investment in the mass of eggs that they had in the first round, even though the attractiveness of their partners had been reversed. There are several possible explanations for this. Firstly, this greater level of investment could be maintained in the laboratory, whereas a trade-off may be more likely in the wild, where resources are limiting.

However, this does not explain why investment in egg mass did not change in relation to male attractiveness. A similar phenomenon has been reported in mice, *Mus musculus*, by Charalambous *et al.* (2003) who found that females experimentally manipulated to invest less in their first litter subsequently also invested less in their second litter. They suggested that females are somehow primed by their experience with their first litter. Several factors may affect this priming effect. Gil *et al.* (1999) used a crossover design and found that female zebra finches did alter their yolk steroid deposition when mate attractiveness was changed with a break of less than a week between breeding

rounds. However, in their experimental design females were left with their mates only for the time it took to lay a single clutch and no chicks were reared. In contrast, in the current study, pairs were together for two months during which time they reared chicks. Perhaps, in the wild females are adapted to alter their investment depending on the perceived quality of their mate only at the start of a breeding season, since mortality between seasons is likely to mean birds frequently have new mates at this time. Once they breed and rear chicks successfully, they will usually stay with the same male and rear another brood (Zann 1996). Therefore they would not need to change their investment in the second breeding attempt. A change in allocation of resources may also be more likely when they abandon a clutch or have it predated and re-lay with a new mate. In Gil *et al*'s experiment, the removal of the first clutch may have had a similar effect to a failed mating attempt in the wild.

Following on from this priming hypothesis, the time interval between breeding attempts may be important. In the current study, in the third round of breeding before which females were given a longer rest interval, there were no differences between the two treatments, suggesting that the colour rings were no longer having any influence on female investment with respect to egg mass (androgen assays were unavailable for this third round).

It is evident from the current study that a female's previous experience may have a profound effect on her reproductive decisions, and needs to be considered in experimental designs of this type. In this study, the first round of breeding seemed to have the stronger effect on female investment. This was unexpected, since recent experience is thought to be important in decision making in zebra finches (Collins 1995) and attractiveness of the second mate would have been increased/decreased relative to the first mate. It is important to note, however, that females were not naïve in the first round since they all had prior breeding experience (although not with colour-ringed males). This means that they should have already possessed a 'bench-mark' for male attractiveness at the start of the experiment.

A final possibility is that there may have been a trade-off within the reproductive attempt (Heaney & Monaghan 1995) so that greater expenditure in terms of egg mass was traded off, for example, against investment during the chick provisioning stage. This may

explain why, at the end of the period of parental care, there was no difference between treatments in the number or quality of offspring. In a study of wild zebra finches, females mated to red-ringed males laid a larger clutch, but did not fledge more young (Zann 1994b). There may be a limit to which females can increase their investment, especially if attractive males are reducing their investment (Burley 1988) and if there is no assortative mating.

### **Offspring quality**

Burley (1986a) found that red-ringed pairs reared more and higher quality offspring (chicks of red-ringed pairs weighed about 5% more at fledging than chicks of green-ringed pairs). However, in the current study, there were no differences in the number or quality of offspring produced by pairs in the two colour treatments. There are obvious fitness benefits to chicks from heavier eggs since heavier eggs were better provisioned with yolk and albumen, and chicks from such eggs were more likely to survive after hatching. However, unexpectedly, embryo mortality was greater for heavier eggs in the red-ring treatment in round one, resulting in there being no difference in mass at hatching between treatments. This may be part of the reason why in round one, when red-ringed females were found to invest more in egg mass, there was no subsequent differences in offspring development between treatments.

There are additional possibilities. The benefits of larger eggs on offspring growth and survival may be more pronounced in harsh conditions compared with in a laboratory environment where food is provided *ad libitum* (Smith & Bruun 1998, Styrsky *et al.* 1999). In the experiments carried out by Burley (1981, 1986a), the more competitive aviary environment may have inflated any differences in offspring development, but assortative mating in her study may also have been the source of such differences. If attractive males mated with higher quality females, then this could have resulted in these females being capable of rearing higher quality offspring. Therefore, both differential allocation and differential access were probably important processes in Burley's experiments, and responsible for the greater reproductive success among red-ringed pairs.

### **Androgen levels**

As mentioned earlier, androgen levels were not affected by ring colour. However, concentrations of T were higher in round one, and this may have been the cause of high embryo mortality in this round. High concentrations of T have been found to correlate with lower hatching success in house sparrows, *Passer domesticus* (Mazuc *et al.* 2003) and to result in reduced nestling growth and survival in American kestrels, *Falco sparverius* (Sockman & Schwabl 2000).

In contrast, other studies have demonstrated that T is beneficial to chicks. For example, it is known to increase begging and growth in canaries (Schwabl 1993, 1996a) and its effects are currently being investigated in zebra finches (L. Gilbert pers. comm.). One explanation to reconcile these conflicting results is that, increased T deposition may be beneficial under most circumstances, promoting chick growth rates. However, when levels are elevated in eggs containing embryos that are not truly 'high quality', then its effects on the chick may be costly (Gil *et al.* 1999). The latter situation would arise in the current experiment, where male attractiveness was artificially altered and where there was no free mate choice.

Alternatively, it has been proposed that females in poorer condition may compensate for the lower quality of their eggs and increase androgen deposition in these clutches (Groothuis & Schwabl 2002). The negative correlation between DHT concentration and female fat score found in the current study suggests that females in poor condition may have employed such a strategy.

### **Sex allocation at laying**

There was no evidence of differential investment in male and female offspring at laying. Male and female eggs did not differ in mass, in agreement with previous zebra finch studies (Clotfelter 1996, Rutkowska & Cichon 2002), or in androgen concentration in either treatment. Nor was there any difference in the primary sex ratio between treatments, nor any tendency for male or female eggs to be preferentially positioned in the laying sequence in either treatment. Zann & Runciman (2003) carried out a similar experiment to this one using semi-domestic finches (5<sup>th</sup> and 6<sup>th</sup> generation wild-caught stock) and also found no evidence of primary sex ratio adjustment.

However, female zebra finches have been shown to facultatively adjust their sex ratio at laying (Rutkowska & Cichon 2002, this thesis Chapter 4) and at hatching (Bradbury & Blakey 1998, Kilner 1998) in response to a poor diet. There may be several reasons for this apparent discrepancy. Under poor dietary conditions, primary sex ratio manipulation is highly advantageous, since females reared on a low quality diet have lower rates of growth and survival compared with males (de Kogel 1997, Bradbury & Blakey 1998, Kilner 1998, Birkhead *et al.* 1999, Martins 2003). However, both sexes may benefit from being fathered by an attractive male. Male variance in reproductive success is probably not much greater than that of females given the low rates of EPCs reported in the wild (Birkhead *et al.* 1990). Furthermore, high quality genes may equally benefit daughters, especially since female survival (de Kogel 1997, Bradbury & Blakey 1998, Kilner 1998) and reproductive success (Haywood & Perrins 1992) are more dependent on nestling conditions than that of males.

Secondly, male quality may be harder for females to assess compared with environmental quality and their own condition (West & Sheldon 2002), in which case, a later mechanism of brood sex ratio adjustment may be advantageous (as suggested by Burley 1986b). Thirdly, attractiveness and parental care can have opposing effects (Burley 1988, Saino *et al.* 1999, Grindstaff *et al.* 2001) and the benefits of producing sons may be balanced by the cost to females of compensating for reduced parental care by attractive males, if sons are more costly to rear (although this is probably not the case in zebra finches). Following on from this idea, the situation may be even more complex. Because attractive males provide less care, a female's investment may also depend on her own body condition, such that only females in good condition invest in the more expensive sex.

Similar patterns of sex ratio adjustment to that found in zebra finches have been reported in other species. In great reed warblers male nestling provisioning was correlated with the sex ratio at hatching but male attractiveness was not (Westerdahl *et al.* 1997, 2000). Similarly, in blue tits female age and nest box location had stronger effects on primary sex ratio bias than did male UV coloration (Sheldon *et al.* 1999).

### Sex ratio adjustment after laying

At first glance, the paired sex ratio data appear to support the studies by Burley (1981, 1986b), since there was no sex ratio bias at laying, but there was a skew towards the more attractive sex at hatching and at fledging. However, the sample size was small, and this, together with the unknown fates of second eggs, means that such a conclusion would be premature. The analyses of the separate breeding rounds complicate the situation. In round one (for which the egg mass data suggest there is a stronger response to the colour rings), there was a difference in the sex ratio at fledging between the treatments (the sex ratio was more female-biased among red-ringed pairs), but this was in the opposite direction to Burley's findings. The mortality data suggest that this is due to higher male mortality among red-ringed embryos prior to hatching. However, in round two, the sex ratio at hatching and at fledging was the opposite of round one (in this round the sex ratio was more female-biased among green-ringed pairs), and thus in line with Burley's predictions. However, this sex ratio bias was already present at hatching, and so not the result of parental manipulation post-hatching as suggested by Burley (1986b). Furthermore, from Table 8 it can be seen that the patterns of embryo mortality in round two are the reverse of round one. Male embryo mortality tended to be higher in the green-ringed treatment, although this did not approach statistical significance. This opposite trend in round two, therefore, would again seem to reflect a carry-over effect.

Male embryo mortality was particularly high in round one. High male mortality was also found in a zebra finch study carried out by Rutkowska & Cichon (2002) and suggests that there is either a sex difference in the provisioning of the eggs or in the sensitivity to resources in the egg. There is evidence that males grow faster than females, at least in poor conditions (Martins 2003), supporting such a hypothesis, although the nature of the sex differences remains unknown. One possibility is that male eggs are provisioned with higher concentrations of yolk steroids or, that they are more sensitive (i.e. possess more receptors) to such steroids. Gil *et al.* (1999) proposed that elevated levels of yolk T could be costly to offspring unless they were of sufficiently high quality. In experiments where male attractiveness is manipulated artificially, females are deceived into perceiving the males as high quality when in fact they may not be. In an aviary, there is the opportunity for assortative mating whereby high quality females may pair with

attractive males, so that the female at least might be of high quality. This was not the case in the current study, where females were of random quality with respect to their mates, and so the offspring may not have been of sufficiently high quality to withstand extra T. Male embryo mortality was higher than that of females in round one (and tended to be more so among red-ringed pairs) and mortality tended to be higher for red-ringed pairs post-hatching. This hypothesis assumes, of course, that androgens are higher for red-ringed pairs. There was no evidence to support this in the current study, although this has been clearly demonstrated in a study using freshly laid eggs (Gil *et al.* 1999). This hypothesis also assumes a sex difference in yolk androgen deposition, for which again there was no evidence in the current study. However, male embryos could be more sensitive to yolk androgens than female embryos, or the incubation of eggs could have concealed differences in initial maternal yolk androgen deposition (Elf & Fivizzani 2002). Leading on from this hypothesis, it is possible that in Burley's study, increased levels of T in eggs of red-ringed pairs resulted in males having a competitive advantage over females and so in the male-biased sex ratio at fledging among red-ringed pairs.

Sex-biased embryo mortality has also been found in other species. In tree swallows (Whittingham & Dunn 2001) mortality was male-biased, but in red-winged blackbirds, differential mortality of female embryos of older females was thought to be responsible for the male-biased sex ratio at hatching (Blank & Nolan 1983). In neither of these species was sex-biased provisioning at laying investigated.

Post-hatching, chicks from heavier eggs had greater survival than chicks from lighter eggs. However, as mentioned earlier, egg mass did not differ between male and female eggs in either treatment, despite this being an obvious mechanism of promoting the fitness of one sex. The feasibility of such a mechanism has been demonstrated in other species (Mead *et al.* 1987, Anderson *et al.* 1997, Cordero *et al.* 2000)

### **Experimental design**

Some of the differences between the results of Burley (1981, 1986a, 1986b) and those in the current study, particularly the sex ratio data, might be because of the different social environments presented by cage and aviary studies. Sex ratio manipulation in relation to male attractiveness may only be beneficial in an environment where both attractive and

unattractive individuals are present in the 'population' for mate comparison. Furthermore, in an aviary environment there are large perceived benefits of producing sons when mated to an attractive male in terms of superior mate acquisition, polygyny and opportunities for EPCs. For example, it has been shown that the population sex ratio of zebra finches affected the importance of the sexually selected beak colour, such that it had a greater influence on reproductive success when females had a greater choice of potential mates (Burley & Calkins 1999). However, there was no sex ratio bias at fledging detected in the aviary study carried out by Swaddle (1996), where both attractive and unattractive individuals were present in the same aviaries. Currently, Burley's original aviary experiment is being repeated on a large scale, involving several research groups to test whether her results can be replicated (N. von Engelhardt, pers. comm.).

Another possibility is that red-ringed males were not perceived as more attractive because in a cage there would be limited opportunity for females to gauge relative attractiveness (although cages were in view of other cages with both types of colour-ringed males visible), and so ring colours would have limited impact. However, since a difference in egg resource allocation was detected, and differences in testosterone deposition have been demonstrated in a similar cage-design experiment (Gil *et al.* 1999), this explanation seems unlikely. It is also important to note that these females had bred previously, and therefore could have made comparison with previous mates.

In the light of other avian studies on sex ratio manipulation in relation to male attractiveness, it is perhaps not surprising that no significant primary sex ratio skew was detected. Very few studies have found convincing evidence for this, and in many field studies there were confounding variables such as territory quality (e.g. Kölliker *et al.* 1999) making it difficult to separate the effects of territory quality and parental phenotype on mating decisions (Halliday 1983). The current study used a within-individual approach to examine primary sex ratio adjustment, as advocated by Oddie & Reim (2002). In a three year study on great tits, they found that females mated to males of better condition relative to their last breeding attempt, tended to bias their brood sex ratios towards males, and females mated with males in relatively worse condition adjusted their brood sex ratios negatively. Such sex ratio adjustment might be easily missed by averaging all individuals in a population (Oddie & Reim 2002, West *et al.* 2002). The current study was designed to

allow a within-individual approach, but the carry-over effect could have invalidated such an analysis.

In conclusion, this study provides evidence for differential allocation at laying in the absence of assortative mating in zebra finches. In terms of the situation in the wild, and probably also in Burley's experimental set-up, it would seem likely that both assortative mating and differential allocation operate, and together influence a female's lifetime reproductive success (de Lope & Møller 1993). This study also highlights the differences that can arise between cage and aviary studies, as well as the influence of experience on female resource allocation.

The data on sex ratio adjustment at laying suggest that there are no benefits to skewing the sex ratio in relation to mate attractiveness. However, the potential complexity of selection pressures and the carry-over effect, means that this cannot be completely ruled out. The sex-biased mortality found pre-hatching in relation to treatment (and perhaps the role of T in this) requires further investigation. Furthermore, in assessing the advantages of sex ratio adjustment, the long-term fitness benefits to females of producing sons and daughters in relation to their own 'quality' as well as that of their mate needs to be addressed.

## **Chapter 3 Appendix: Aviary pilot study**

### **Introduction**

In order to provide a closer comparison with the studies by Burley (1981, 1986a, 1986b), an aviary experiment was carried out. As well as providing a more natural environment, aviaries give some opportunity for mate choice. However, there was an important difference in the set-up between these aviaries and those in Burley's study. Burley's aviaries contained both red- and green-ringed males within a single aviary, but in the experiment described below, aviaries contained males of only one ring colour, so that assortative mating between colour-ringed birds was not possible. The aviary experiment is presented as a pilot study since, due to vandalism, the aviaries had to be decommissioned, and replicates could not be performed, resulting in a limited data set.

### **Methods**

#### **Experimental design**

The experiment was carried out in June and July 2001. Twenty-four males were divided into two groups. Twelve males formed the 'attractive' group and were given a red ring on each leg. Twelve males formed the 'unattractive' group and were given a green ring on each leg. Males were then split into four groups and released into outdoor aviaries, each measuring 2 x 4 x 2m, so that aviaries 1 and 3 contained six red-ringed males each, and aviaries 2 and 4 contained six green-ringed males each. In addition to their coloured-leg rings, males were also fitted with one or two 'neutrally-coloured' orange leg rings, arranged in a unique combination so that individuals could be distinguished from the window situated at one end of the aviary. Six female zebra finches were then released into each aviary. Females were fitted with orange leg rings only, in unique combinations again to enable individual recognition. The four aviaries were positioned in a line, adjacent to one another, in visual and acoustic contact with one another.

Each aviary was equipped with eight closed-design nest boxes to provide some shelter from the elements, and abundant nesting material. Foreign finch mix and fresh

water were available *ad libitum*. Haith's egg mix and fresh spinach were provided three times a week.

Nests were checked for eggs and chicks as described in Chapter 2. The male and female associated with each nest were identified by their leg rings, and reported as the putative parents when they had been observed at least twice attending the nest during the incubation period. Any chicks that died, and any unhatched eggs containing embryos, were removed for molecular sexing (see Chapter 2).

After the last chick had fledged from a nest, the nests were checked daily for second clutch eggs, which were numbered and weighed as before. Nests were observed to confirm the identity of the parents, as a pair did not always use the same nest box to lay their second clutch. Eggs were candled to check for the presence of an embryo two days after the first egg of a second clutch had been laid. Once an embryo was sufficiently large for sexing, the egg was removed, replaced with a dummy egg, and then taken to the laboratory for sexing.

## Results

### Clutch size and egg mass

Clutches were laid by seven red-mated females (four from aviary 1 and three from aviary 3) and nine green-mated females (four from aviary 2 and five from aviary 4), although one of the green-ringed nests was laid in the aviary wall and not discovered until late in the incubation period, and so not included in the clutch analyses below. There were no significant differences in clutch size, mean egg mass, or mean brood size between red- and green-ringed pairs (Table 1).

In a repeated measures GLM, where female and aviary were both entered as random factors, there was no difference in egg mass between females mated to red- and green-ringed males ( $F_{1,14} = 1.70, p = 0.21$ ). Females were not weighed, so this covariate could not be entered into the model. Egg mass increased significantly with position in the laying sequence in both treatments ( $F_{1,80} = 17.92, p < 0.0001$ , Fig. 1). There was no difference in the mass of male and female eggs ( $F_{1,52} = 0.34, p = 0.56$ ) and the interaction between ring colour and embryo sex with respect to egg mass was not significant ( $F_{1,51} = 0.29, p = 0.60$ ).

Table 1. Clutch data for all four aviaries. Differences in days to laying and clutch size were analysed using a Mann-Whitney U test. Differences in mean egg mass per clutch and mean brood size at fledging were analysed using an independent samples t-test.

|   | <b>Red pairs n = 7</b> | <b>Green pairs n = 8</b> |                                  |
|---|------------------------|--------------------------|----------------------------------|
| <b>Median day to 1<sup>st</sup> egg (IQR)</b> | 6 (5-7)                | 5.5 (4.25-6)             | W = 64.5, p = 0.35               |
| <b>Median clutch size (IQR)</b>               | 5 (5-6)                | 6 (5.25-7.5)             | W = 45.5, p = 0.25               |
| <b>Mean egg mass per clutch g (S.E)</b>       | 1.24 (0.05)            | 1.30 (0.03)              | T <sub>10</sub> = 0.96, p = 0.36 |
| <b>Mean brood size at fledging (S.E)</b>      | 3.8 (0.95)             | 4.2 (0.55)               | T <sub>8</sub> = 0.27, p = 0.8   |

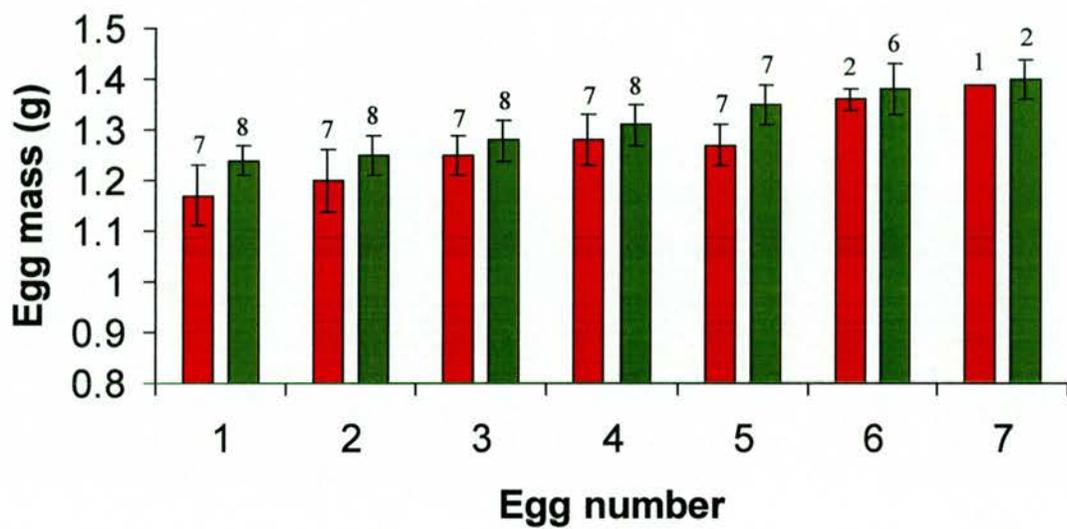


Figure 1. Mean egg mass ( $\pm$  SE) at each position in the laying sequence for females mated to red-ringed males ( $n = 7$ , shown by red bars) and females mated to green-ringed males ( $n = 8$ , shown by green bars). Numbers of eggs are given above bars.

### Sex ratio

The sex of all eggs and chicks produced in each treatment is summarized in Table 2. There was no difference in the mean clutch sex ratio at laying (number of males/ total number of sexed eggs) between the two treatments (mean clutch sex ratio  $\pm$  SE, red-ringed pairs:  $0.46 \pm 0.09$ ,  $n = 7$ , green-ringed pairs:  $0.34 \pm 0.07$ ,  $n = 9$ ,  $F_{1,14} = 0.35$ ,  $p = 0.56$ ). There was no difference in the clutch sex ratio when second clutches were included in the analysis (red-ringed pairs:  $0.49 \pm 0.08$ ,  $n = 11$ , green-ringed pairs:  $0.47 \pm 0.06$ ,  $n = 13$ ,  $F_{1,14} = 0.10$ ,  $p = 0.74$ ). There was also no difference in the mean brood sex ratio at hatching (red-ringed pairs:  $0.52 \pm 0.09$ ,  $n = 6$ , green-ringed pairs:  $0.39 \pm 0.08$ ,  $n = 9$ ,  $F_{1,13} = 1.13$ ,  $p = 0.30$ ). Finally, there was no difference in the sex ratio at fledging (red-ringed pairs:  $0.58 \pm 0.09$ ,  $n = 5$ , green-ringed pairs:  $0.38 \pm 0.09$ ,  $n = 9$ ,  $F_{1,12} = 1.88$ ,  $p = 0.20$ ).

In both colour treatments the sex ratio tended to decrease with position in the laying sequence, i.e. become increasingly female-biased ( $F_{1,60} = 4.26$ ,  $p = 0.04$ , Fig. 2) although, when the analysis was carried out separately for each ring colour, this was only significant for green-mated females ( $F_{1,35} = 5.08$ ,  $p = 0.03$ ), not for red-mated females ( $F_{1,24} = 0.35$ ,  $p = 0.56$ ). When second clutches were included in the analysis, there was no significant effect of laying sequence on embryo sex in either ring colour treatment ( $F_{1,96} = 2.46$ ,  $p = 0.12$ ).

### Hatching success and mortality

Young hatched from all nests, and embryonic mortality was very low, only 6.8% (5/73) of eggs, from four nests, failed to hatch. Unsurprisingly, given this low mortality rate, embryo mortality was not significantly affected by ring colour ( $F_{1,12} = 0.06$ ,  $p = 0.82$ ), position in the laying sequence ( $F_{1,50} = 0.03$ ,  $p = 0.87$ ), embryo sex ( $F_{1,51} = 0.71$ ,  $p = 0.40$ ) or egg mass ( $F_{1,52} = 3.28$ ,  $p = 0.08$ ). Post-hatching mortality was also low, 16.2% (11/68) of chicks died, from six nests. Again, post-hatching mortality was not affected by ring colour ( $F_{1,12} = 0.09$ ,  $p = 0.77$ ), laying sequence ( $F_{1,46} = 0.00$ ,  $p = 0.99$ ) embryo sex ( $F_{1,47} = 0.11$ ,  $p = 0.74$ ) or egg mass ( $F_{1,48} = 0.70$ ,  $p = 0.41$ ). There was no interaction between ring colour and chick sex with respect to chick mortality ( $F_{1,50} = 0.91$ ,  $p = 0.34$ ).

Table 2. The number of chicks of each sex at laying, hatching and fledging for females mated to red- and green-ringed males. Sex ratio = males / (males + females). The numbers in brackets include second clutches.  $G_{adj 1}$  values are goodness of fit against the Mendelian expectation of equal numbers of both sexes. M = males, F = females.

|                   | Laying     |                  |                | Hatching       |            |       | Fledging  |             |            |       |           |             |
|-------------------|------------|------------------|----------------|----------------|------------|-------|-----------|-------------|------------|-------|-----------|-------------|
|                   | No. broods | M/F              | Sex ratio      | $G_{adj 1}$    | No. broods | M/F   | Sex ratio | $G_{adj 1}$ | No. broods | M/F   | Sex ratio | $G_{adj 1}$ |
| Red-ringed mate   | 7<br>(11)  | 15/17<br>(23/26) | 0.47<br>(0.47) | 0.12<br>(0.18) | 6          | 14/12 | 0.54      | 0.15        | 5          | 13/9  | 0.59      | 0.72        |
| Green-ringed mate | 8<br>(13)  | 20/30<br>(32/44) | 0.40<br>(0.42) | 1.99<br>(1.89) | 9          | 18/27 | 0.40      | 1.79        | 9          | 15/23 | 0.39      | 1.67        |

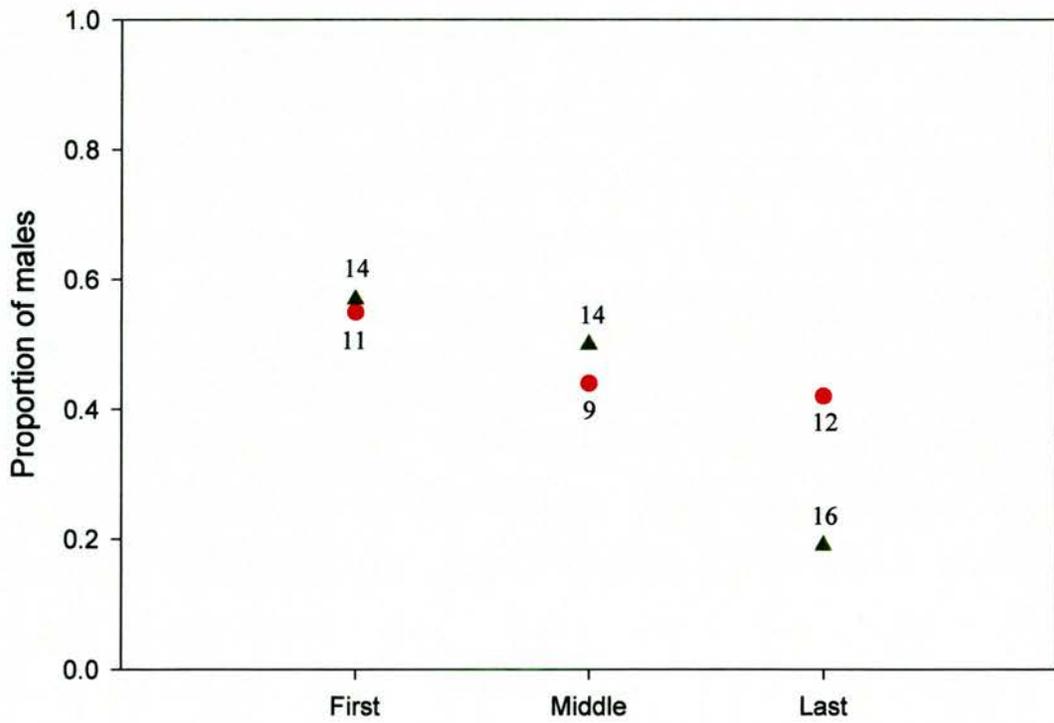


Figure 2. Sex ratio (proportion of males) in relation to relative position in the clutch (first, middle or last) for first clutch eggs, laid by red- and green-mated females, shown by red circles and green triangles, respectively. Numbers beside points show the number of eggs that were sexed.

### Offspring development

There was no effect of ring colour on hatchling mass, fledgling mass, fledgling tarsus length, or fledgling age (Table 3). However, as adults, offspring of red pairs tended to be heavier (mean  $\pm$  SE,  $18.34 \pm 0.43$ g,  $n = 15$ ) than offspring of green pairs ( $17.13 \pm 0.30$ g,  $n = 18$ ), but offspring of green pairs had longer tarsi ( $13.9 \pm 0.07$ mm) than offspring of red pairs ( $13.6 \pm 0.10$ mm). There were no effects of laying sequence or chick sex on any of the offspring measurements (Table 3).

### Discussion

The sample sizes from this experiment are insufficient to provide meaningful conclusions concerning differential allocation at laying or differential investment in male and female offspring. Differences in adult mass and tarsus length between the treatments suggested that replication on a larger scale would be worthwhile. Differences in offspring development may be more apparent in an aviary environment because there is more competition between chicks and the harsher, outdoor climate may mean that fitter chicks out-compete less fit chicks.

The results revealed some interesting comparisons with the main cage experiment. Hatching success was significantly higher in the aviaries compared with the individual cages, which is a common phenomenon in zebra finches (D. Gil pers. comm.). It has also been found in other avian species that, females with no mate choice produce fewer, lower quality offspring compared with females given the opportunity to choose a mate (Bluhm 1985, Yamamoto *et al.* 1989). The lower reproductive success of females with no mate choice could itself be due to lower reproductive effort (de Lope & Møller 1993). Female field crickets, *Gryllus bimaculatus*, that were allowed to choose their mates laid larger clutches than females that were allocated mates, and the young from such eggs developed at a faster rate and had higher survival (Simmons 1987). Although, in the current study, females laid clutches and eggs of a similar size to those in individual breeding cages, the eggs may have differed in quality, or the females may have incubated them for longer periods. The higher reproductive success in the aviaries could also be due to less disturbance from animal house technicians. However, this difference in reproductive

Table 3. Factors affecting offspring development. N = 7 red-ringed pairs and 8 green-ringed pairs.

| Trait                | Ring colour |      | Laying sequence |      | Sex  |      | Egg mass |      |      |      |       |           |
|----------------------|-------------|------|-----------------|------|------|------|----------|------|------|------|-------|-----------|
|                      | df          | F    | p               | df   | F    | p    | df       | F    | p    |      |       |           |
| Hatching mass (g)    | 1,17        | 0.01 | 0.94            | 1,47 | 0.49 | 0.49 | 1,55     | 1.29 | 0.26 | 1,33 | 71.04 | <0.0001** |
| Days to fledging     | 1,15        | 1.05 | 0.32            | 1,44 | 0.00 | 0.95 | 1,45     | 3.02 | 0.09 | 1,37 | 2.08  | 0.16      |
| Fledging mass (g)    | 1,13        | 1.48 | 0.25            | 1,53 | 1.12 | 0.29 | 1,41     | 0.36 | 0.55 | 1,32 | 2.48  | 0.12      |
| Fledging tarsus (mm) | 1,17        | 1.55 | 0.23            | 1,40 | 1.18 | 0.28 | 1,53     | 1.90 | 0.17 | 1,25 | 0.64  | 0.43      |
| Adult mass (g)       | 1,16        | 4.16 | 0.059           | 1,47 | 0.23 | 0.64 | 1,44     | 0.12 | 0.73 | 1,30 | 0.00  | 0.99      |
| Adult tarsus (mm)    | 1,16        | 4.60 | 0.048           | 1,41 | 0.03 | 0.86 | 1,47     | 0.73 | 0.40 | 1,25 | 0.01  | 0.92      |

success has also been found in the case where females have been able to choose their mates in an aviary, but breeding has taken place in cages (pers. obs.).

## **Chapter 4: Diet-dependent differences in reproductive investment**

### **Introduction**

#### **Resource allocation**

Lack (1968) postulated that, for most birds reproduction is ultimately controlled by food availability because breeding occurs at times of year at which food supply appears at a maximum. Many studies have since demonstrated that diet and maternal condition are key factors in determining reproductive investment and success in birds (Houston *et al.* 1983, Bolton *et al.* 1992, Bolton *et al.* 1993, Potti 1993, Smith *et al.* 1993, Gorman & Nager 2003, Wendeln & Becker 1999).

Females are predicted to adjust their investment to maximise their long-term reproductive success. Therefore, when resources are plentiful, females should make relatively large reproductive investments, as reflected by clutch and egg size. Laying larger eggs is advantageous since chicks that hatch from larger eggs have higher growth rates (Perrins 1965, Schifferli 1973) and better survival prospects (Parsons 1970, Nisbet 1978, Lundberg & Vaisanen 1979, O'Connor 1979, Heaney & Monaghan 1995). Egg size may also vary considerably within clutches and, in asynchronously hatching species the relative size of later eggs may influence the survival prospects of last hatching chicks (Slagsvold *et al.* 1984).

#### **Sex allocation**

Diet and maternal condition are also important factors linked to sex ratio adjustment. Sex allocation theory predicts that parents should invest more in the sex that yields the highest fitness returns (Trivers & Willard 1973, Charvov 1982). This may be achieved in several ways. Females may differentially allocate resources to male and female eggs (Anderson *et al.* 1997, Cordero *et al.* 2000, 2001, Petrie *et al.* 2001, Magrath *et al.* 2003), preferentially feed chicks of one sex (Stamps *et al.* 1987, Yasukawa *et al.* 1990) or, produce unequal numbers of male and female ova. In their maternal condition hypothesis, developed for a polygynous mammalian breeding system, Trivers & Willard (1973) proposed that a female

in good condition should produce sons since she will leave more surviving grandchildren than if she produced daughters, while the reverse is true for an adult female in poor condition. Their model assumed that female condition correlated with offspring condition at the end of the period of parental investment, and that these differences were maintained into adulthood. It also assumed that adult differences in condition affected male reproductive success more strongly than they affected female reproductive success. Their model has since been modified to encompass other types of mating systems and environmental conditions.

Alternatively, Myers (1978) proposed that the costs associated with rearing sexually dimorphic offspring may be a more important influence on parental manipulation of offspring sex ratios. Parents could produce more young in an unpredictable environment by investing in the cheaper sex during periods of food stress and investing in the more costly sex during periods of resource abundance.

Avian skewed sex ratios at fledging may be the result of sex ratio adjustment at a primary level, i.e. at ovulation (Krackow 1995), and/or at a secondary level, through sex-biased mortality. The latter could be brought about through direct parental manipulation by selective provisioning of one sex or, through differential vulnerability of one sex to lower parental investment or unfavourable rearing conditions (Clutton-Brock 1991). Sex-biased mortality in size dimorphic species may favour the larger sex, which can dominate and out-compete the smaller for food (Smith *et al.* 1989, Teather 1992, Oddie 2000) or, alternatively, the larger sex may be the first to starve when food is limited (Howe 1976, Røskaft & Slagsvold 1985, Weatherhead & Teather 1991, Griffiths 1992) because of higher food demands (Anderson *et al.* 1993).

In line with the predominant theories of sex allocation, sex ratio skews in relation to resource availability have been well documented in species with pronounced sexual size dimorphism, resulting in the larger sex being more costly to rear. For example, experimental studies on the lesser black-backed gull, *Larus fuscus*, a species in which males are larger and grow faster than females, have shown that sex biases towards daughters in broods where females are in poor condition are the result of a combination of mechanisms. Nager *et al.* (1999) manipulated maternal condition and found that as a female's condition deteriorated, resulting in the production of lower quality eggs, she

progressively skewed the sex ratio of her eggs towards the smaller sex, females. When eggs were cross fostered, males from poor quality eggs were found to have higher mortality than females from poor quality eggs. In a further experiment (Nager *et al.* 2000a) female condition was manipulated and parents were given a control clutch to rear. Pre-fledging survival of male chicks was reduced when they were reared by parents in poor condition, especially in all male broods, whereas condition of female chicks was unaffected by parental composition or the brood sex composition. Therefore, a production bias at laying and a differential effect of egg quality and rearing environment on male survival could bring about sex ratio skews in relation to female condition.

That this skew towards females when in poor condition was an adaptive mechanism rather than a general physiological constraint, was supported by a study on the great skua, *Catharacta skua*, a species with reversed sexual size dimorphism (females are larger than males). Females that were manipulated to produce more eggs, produced more males at the end of the laying sequence compared with control females whose condition was maintained by supplementary feeding during laying (Kalmbach *et al.* 2001).

In contrast, when food is abundant the more expensive sex or the sex whose reproductive success is more dependent on nestling resources, should be produced. In tawny owls, *Strix aluco*, more female-biased clutches were produced on territories with higher vole densities (Appleby *et al.* 1997). This was thought to be adaptive since there was a significant relationship between the number of chicks fledged by adult females and the vole abundance in the territory on which they were reared as chicks, but no such relationship existed for males.

Seasonality may also influence the survival and reproductive opportunities of the sexes in different ways because of differential effects of fledging date on the probability of early breeding for each sex. A seasonal decrease in the proportion of sons in the European kestrel, *Falco tinnunculus*, is probably adaptive because early fledging sons but not daughters are more likely to breed as yearlings (Dijkstra *et al.* 1990b). The opposite is true for crimson rosellas, *Platycercus elegans*. Yearling females, but not males are more likely to breed, so the female-biased sex ratio at the start of the season reflects the differential influence of fledging date on male and female fitness (Krebs *et al.* 2002).

In polygynous species, females in good condition are predicted to produce male-biased broods. In the polygynous great reed warbler, *Acrocephalus arundinaceus*, the brood sex ratio was related to the status of the female, which predicted how much help she received from the male (Westerdahl *et al.* 2000). Sexing of nine-day-old chicks revealed that broods of females of primary harem status comprised a higher proportion of sons than broods of secondary females because males predominantly assist primary females. In this species, males are significantly heavier than females and have higher variance in reproductive success. Similarly, in the polygynous house wren, *Troglodytes aedon*, second-mated females who receive little or no parental assistance from their mate, and consequently fledge fewer and lower quality young than first-mated females, produced female-biased broods (Albrecht & Johnson 2002).

Higher variance in male reproductive success is not just confined to polygynous species. In many socially monogamous species extra-pair fertilisations are common and may result in much greater variance in male reproductive success than previously thought (Birkhead & Møller 1992, Webster *et al.* 1995, Dunn & Cockburn 1999). For example, tree swallows, *Tachycineta bicolor*, are socially monogamous but have one of the highest known levels of extra-pair paternity in birds, with 38 - 76% extra-pair young (Dunn *et al.* 1994), and so variance in male reproductive success is substantially greater than that of females. Accordingly, male-biased primary sex ratios were associated with females in better body condition, and these females were more likely to produce sons in better condition (Whittingham & Dunn 2000).

In addition to whole brood sex ratio manipulation, it may also be adaptive to adjust the within brood sex ratio, i.e. the relative position of male and female eggs within the laying sequence. This is because the fitness returns for each sex differ depending on their position within the laying sequence. In the polygynous house wren, the last egg in the clutch was the target of sex ratio manipulation. Last-hatched young that consequently fledged in poorer condition tended to be female (Albrecht 2000). The fitness consequences of within brood sex manipulation may be especially pronounced in raptor species where there is usually considerable sexual size dimorphism, accompanied by asynchronous hatching, which can potentially result in large competitive asymmetries within the clutch. Producing the larger sex first may facilitate brood reduction, whereas producing the larger

sex later in the laying sequence will minimise competitive asymmetries between chicks but may increase sibling competition (Edwards and Collopy 1983, Bortolotti 1986). In bald eagles, *Haliaeetus leucocephalus*, females weigh about 25% more than males, but males grow more quickly than females. The combination, male female, which would have resulted in the largest size asymmetry early on, was avoided when food was plentiful, but when food availability was low, this sequence was more prevalent, possibly to encourage efficient brood reduction (Bortolotti 1986, Dzus *et al.* 1996). Similarly, in Harris' hawks, *Parabuteo unicinctus*, there was a highly significant skew towards the smaller sex, males, in first-hatched nestlings, and these nests fledged significantly more young than nests in which females hatched first (Bednarz & Hayden 1991).

### **Diet and zebra finches**

The current chapter is concerned with resource allocation in zebra finches, *Taeniopygia guttata*, in relation to diet quality. In order to make predictions concerning sex ratio manipulation in zebra finches under different dietary conditions, it is necessary to first understand how condition early in life may differentially affect the future productivity of sons and daughters.

Zebra finches are socially monogamous with bi-parental care (Morris 1954, Burley 1988) and therefore, mate choice is important for both males and females (Burley 1985, Burley & Coopersmith 1987, Wynne & Price 1993). The rate of extra-pair copulations (EPCs) in the wild is estimated at only 2-3%, based on a study of a single population (Birkhead *et al.* 1990), but probably depends to some extent on the degree of synchrony within the breeding colony (Zann 1996). However, it has been shown that laboratory females mated to unattractive males will engage more readily in EPCs with more attractive males (Houtman 1992, Burley *et al.* 1994, 1996), suggesting that there may be greater fitness benefits for high quality males than for high quality females.

As to which sex is more affected by resources received as nestlings, the evidence is at first sight equivocal. There is no discernible size dimorphism in this species, although male wing length is about 1% greater than that of females (Zann 1996). For both males and females, adult mass was strongly correlated with growth rates as nestlings and fledging mass (Boag 1987, Skagen 1988). For females, fecundity (clutch size and egg

mass) was strongly related to their mass at 45 days post hatching (Haywood & Perrins 1992), although, attractiveness of males, but not of females was influenced by brood size (de Kogel & Prijs 1996).

Females, however, are more vulnerable to nutritional stress than males. In the field, sex ratios were male-biased when food was limited (Zann & Runciman 1994, Burley *et al.* 1989) and female mortality was found to be greater than that of males when reared under poor conditions both in the field (Zann & Runciman 1994) and in the laboratory (Bradbury & Blakey 1998, Kilner 1998, Birkhead *et al.* 1999). Mortality rates are correlated with fledging weight, and females reared in experimentally enlarged broods fledged at lower weights and experienced higher mortality after independence than males reared in experimentally enlarged broods (de Kogel 1997).

The reason why female chicks are more vulnerable than males to impoverished food may reflect either sex differences in growth rates in relation to resources received (Richter 1983), selective provisioning of male chicks by parents when food is limited (Howe 1977, 1979, Stamps *et al.* 1987) and/or, the ability of males to out-compete females for food in the nest (Oddie 2000). This question was partly resolved by Martins (2003) in a hand-rearing experiment, which eliminated sibling-sibling and parent-offspring interactions. Male and female chicks were raised on different quantities of food from 5-10 days. On low food levels, females gained mass more slowly than males, therefore suggesting one mechanism that might be responsible for female-biased mortality. Importantly, her work illustrated that sex ratio adjustment was not (purely) the result of directed parental chick provisioning. Additionally, her work has shown that there may be sex-specific rearing costs in a size monomorphic species, where costs have been previously assumed to be equal (Albrecht 2000). The cause of these differences in growth rates is not currently known. One possible candidate is a difference in the amount of or effect of yolk androgens in male and female eggs (investigated in Chapter 5). Other possibilities include sex differences in metabolic rates, or in the allocation of energy to different metabolic activities (Richter 1983).

Whatever the mechanism(s), these studies suggest that female condition is more dependent on resources received as nestlings than males, and that, being of lower quality has stronger negative effects on the future reproductive success of females than that of

males. As such, females would seem to be the more costly sex. Three experiments have specifically tested sex allocation in relation to diet in laboratory zebra finches. Kilner (1998) examined the sex ratio at hatching and at fledging in relation to food abundance. She found that when food was restricted, the sex ratio at hatching and at fledging was male-biased, but when food was abundant, the sex ratio was unbiased. In addition, mortality was female-biased on the restricted diet. Under both conditions, females were found to hatch significantly earlier within the brood than males, and first-hatched chicks fledged at higher weights than those that hatched last. She suggested, therefore, that female reproductive success was more condition-dependent than that of males and that the observed sex ratio adjustment was adaptive.

Bradbury & Blakey (1998) examined the hatching and fledging sex ratio in relation to diet quality. Unmated zebra finches were fed for three months on high or low quality diets. Birds were then allowed to breed in two aviaries while keeping on the same diets. They found that females but not males on the high quality diet were significantly leaner and had lower muscle scores than those fed on the low quality diet. Females on the lower quality diet (found to be in better condition) hatched significantly more males than females, whereas females on the higher quality diet hatched an equal sex ratio. Female mortality was also found to be greater than male mortality on the low quality diet. They concluded that production of males was more costly (given their slightly larger size), and so females in good condition should produce males. They suggested that females were reacting more to their own body condition rather than the environmental conditions that their offspring would later have to face.

Rutkowska & Cichon (2002) specifically tested whether female zebra finches adjusted the sex of eggs according to the amount of resources available. Females were given a high or low quality diet for seven weeks. After females had laid their first egg, the diets were reversed. Females that were on a high quality diet and then subsequently given a low quality diet tended to produce more male eggs, suggesting that females were indeed adjusting the sex of their eggs in relation to the resources in the egg.

A fourth study looked at sex ratio manipulation in a population of wild zebra finches whose diet had been supplemented with seed, to investigate whether this would result in a female-biased sex ratio (Zann & Runciman 2003). However, the primary sex

ratio did not differ from parity, providing no evidence that females skewed the sex ratio towards the more costly sex when conditions were favourable. No study to date has found a female sex ratio bias under favourable dietary conditions, and Zann & Runciman did not test whether a poor diet led to a male sex ratio bias in the wild.

### **Aims**

The aim of the current chapter was to investigate the existence of adaptive strategies in relation to diet quality in zebra finches. Diet quality may vary in the wild since zebra finches feed on half-ripe as well as fully ripe seeds during breeding, and these half-ripe seeds may contain nutrients, particularly certain amino acids, important in egg production and chick rearing that are lacking in ripe seed (Zann *et al.* 1995, Allen & Hume 1997).

In the current study, females were given their specified diet for a considerable period (six weeks) prior to breeding, in order that they would perceive the environment as relatively stable, and hence be more likely to adopt different strategies on the two dietary regimes. On a high quality (HQ) diet, females would incur lower costs since the protein required for egg production would be provided by diet rather than from endogenous reserves (Houston *et al.* 1995, Cottam *et al.* 2002). In addition they should be able to rear more and higher quality offspring, and so their overall investment was predicted to increase. I also predicted that, if female fitness is more sensitive than that of males to resources received during the nestling period, then investment in females would be greater on a HQ diet, and investment in males would be greater on a low quality (LQ) diet. Finally, I predicted sex differences in chick development and mortality on the two diets, in accordance with females faring badly on a LQ diet.

Soon after the experiment was begun, it became evident that limited data would be obtained from the LQ diet birds since none of their eggs hatched. For that reason, the experiment was stopped prematurely after the first round of breeding. The results of this experiment are given in the appendix at the end of the chapter. It was decided to increase the quality of the LQ diet slightly to improve the breeding success. In order to maintain a substantial difference between the two diets, the quality of the HQ diet was also increased. A new set of females was used for this second attempt, and the design of the second

experiment was a paired crossover design, such that the resource allocation of individual females could be compared on both diets.

## Methods

### Experimental design

Thirty-four females were weighed and randomly assigned to one of two diet groups. The HQ diet comprised foreign finch mix *ad libitum* (approx. 14% protein), supplemented with daily portions of Haith's egg biscuit (13.2% protein), mixed with hard-boiled hens' eggs (which have an amino acid composition very similar to that of zebra finch eggs; Murphy 1994b), PBX (26% protein crumb) and fresh spinach. Females on the LQ regime were given foreign finch mix *ad libitum*, supplemented with fresh spinach twice a week. Both groups had access to cuttlefish bone, oyster shell grit, and fresh water *ad libitum*. Females were kept on these diets in single sex groups for a period of six weeks, after which they were re-weighed and paired randomly with males with whom they had not previously bred. The pairs were moved into individual breeding cages, equipped with open-design nest boxes and supplied with nesting material. For the six weeks prior to pairing, all males had been caged in a separate room, on the standard laboratory diet of foreign finch mix, supplemented with spinach and egg biscuit once a week. Once paired, males were then kept on the same diet as their mate, and these diets were maintained during chick rearing.

Nests were checked for eggs and hatchlings as described in Chapter 2. Pairs were then permitted to lay second clutches to obtain additional clutch data. Second clutch eggs were weighed and numbered. Two days after the first egg of a second clutch had been laid eggs were candled for embryos. Once an embryo was clearly visible, (usually two-three days after laying), the egg was removed and replaced with a dummy egg. The embryo was extracted and stored at -20°C in 96% alcohol for later molecular sexing.

After the pairs that had laid a second clutch had completed laying, all pairs were weighed and split up and held in single sex groups as before. Birds were given a two-week rest period (during which all females received the standard laboratory diet) before the females were put on the reverse diets. Thereafter, females that were previously on a HQ diet were given a LQ diet, and vice-versa. The males were all once again given the standard laboratory diet. After six weeks, all birds were re-weighed, and re-paired with the

same mate as in the first round of breeding, and the experiment repeated. The mates of any males that died during or after the first round of breeding were excluded in the second round of breeding.

### **Sexing**

DNA was extracted from incubated second-clutch eggs, as well as chicks that died prior to sexual maturity, and eggs that were fertile but failed to hatch from first clutches, using the Puregene technique (see Chapter 2). PCR sexing was carried out as described in Chapter 2. Offspring that survived to sexual maturity were sexed from plumage characteristics.

### **Statistical analysis**

Paired analyses were performed for females that laid clutches in both rounds of breeding. Analyses of egg mass and offspring development data were carried out using repeated measures general linear models (GLMs) as described in Chapter 2. Sex ratio and mortality data were analysed using generalised linear models as described in Chapter 2.

## **Results**

### **Clutch size and egg mass**

Of the 33 females (one female fell ill whilst on the HQ diet and was excluded from the experiment), 20 females laid a clutch on both the HQ and LQ diets (12 females laid on the HQ diet and eight females laid on the LQ diet in round one). Five females did not lay a clutch on either diet, and eight females laid a clutch on only one of the diets. Of these, five laid only on the HQ diet and three laid only on the LQ diet

Paired analyses were carried out on the 20 females that laid a clutch on both diets. There was no difference in the median number of days between pairing and laying of the first egg (Wilcoxon signed ranks test,  $W = 55.0$ ,  $p = 0.53$ ) but on the HQ diet females laid significantly larger clutches (Wilcoxon signed ranks test,  $W = 171.0$ ,  $p < 0.001$ ) and heavier eggs (paired t-test,  $t = 8.23$ ,  $p < 0.001$ ) than when on the LQ diet (Figs. 1a & b). Mean egg mass per clutch was  $13.64 \pm 1.73\%$  heavier (mean  $\pm$  SE) on the HQ diet.

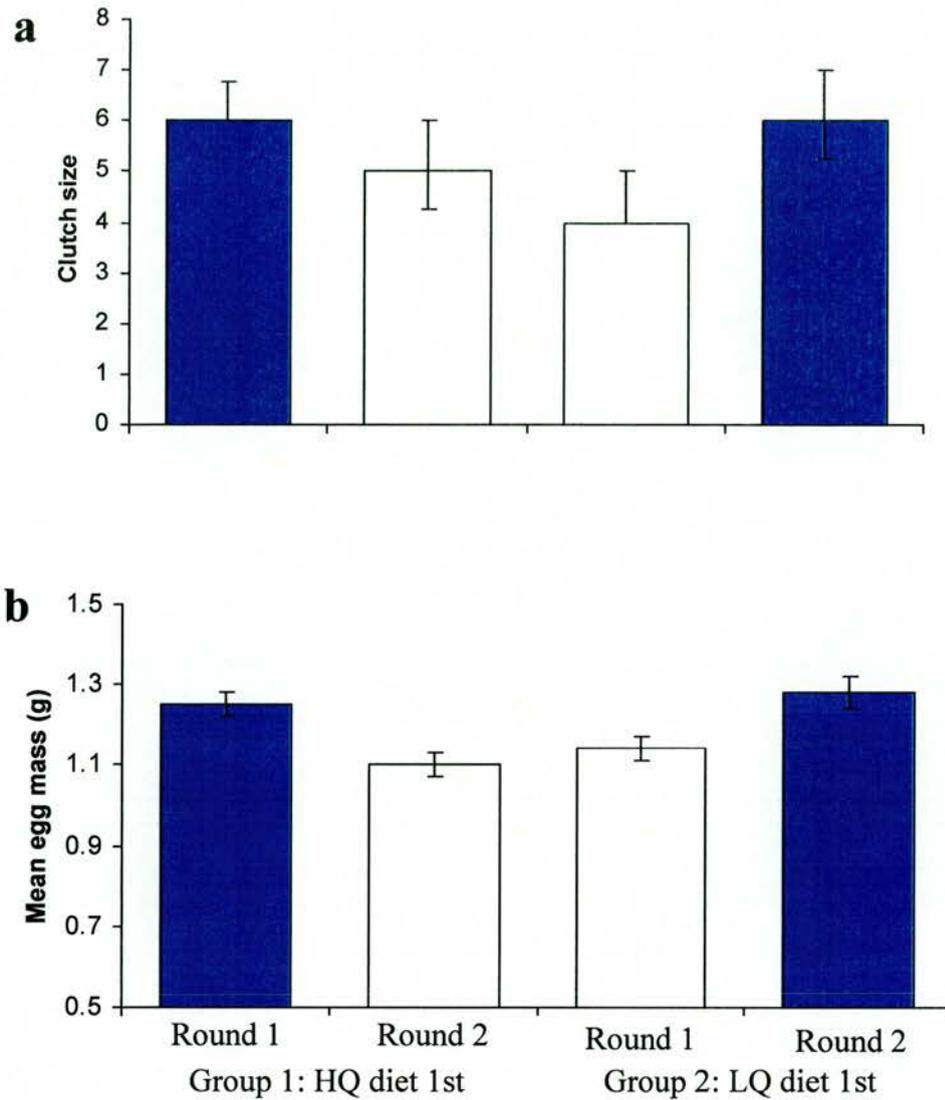


Figure 1. Paired laying data for females that laid a clutch on both the HQ and LQ diets. Figures show (a) median clutch sizes with interquartile ranges, and (b) mean egg mass per clutch  $\pm$  SE. The blue bars represent the HQ diet eggs and the white bars represent the LQ diet eggs. Group 1 females received the HQ diet in round one and the LQ diet in round two ( $n = 12$ ) and Group 2 females received the LQ diet in round one and the HQ diet in round two ( $n = 8$ ).

### **Egg mass and laying sequence**

Egg mass data were analysed in a paired, repeated measures GLM. There was a significant interaction between diet and position in the laying sequence with respect to egg mass ( $F_{1,25} = 21.27$ ,  $p = 0.0001$ , Fig. 2). In the HQ group, egg mass increased significantly with position in the laying sequence ( $F_{1,101} = 16.72$ ,  $p < 0.0001$ ), but egg mass tended to decrease with position in the laying sequence on the LQ diet ( $F_{1,89} = 3.39$ ,  $p = 0.07$ ). There was no effect of order of treatment on egg mass (i.e. whether females were given the HQ or LQ diet first, interaction between breeding round and diet:  $F_{1,36} = 0.32$ ,  $p = 0.58$ ) and no effect of female mass on egg mass ( $F_{1,37} = 2.68$ ,  $p = 0.11$ ). There was no difference in the mass of male and female eggs ( $F_{1,95} = 0.25$ ,  $p = 0.56$ ) and the interaction between diet and embryo sex was not significant ( $F_{1,89} = 0.02$ ,  $p = 0.88$ ).

### **Second clutches**

Fourteen HQ diet females (but no LQ diet females) laid second clutches in round one. Median clutch size did not differ between first and second clutches (median (interquartile range), first clutches: 6 (6 - 6.25), second clutches: 7 (6 - 8.25), Wilcoxon signed rank test,  $W = 10.5$ ,  $p = 0.17$ ), but second clutch eggs were significantly heavier than first clutch eggs ( $F_{1,44} = 5.09$ ,  $p = 0.03$ , Fig. 3a).

In round two, eight HQ and nine LQ diet females laid second clutches. There was an interaction between diet and clutch number with respect to egg mass ( $F_{1,49} = 4.88$ ,  $p = 0.03$ ). The HQ females laid significantly heavier eggs in their second clutch ( $F_{1,30} = 6.86$ ,  $p = 0.01$ , Fig. 3b), but there was no difference in the mass of eggs laid by LQ females between first and second clutches ( $F_{1,14} = 0.10$ ,  $p = 0.75$ , Fig. 3c). Again, clutch sizes did not differ between first and second clutches (median (interquartile range), HQ diet first clutches: 6.5 (6 - 7), second clutches: 6 (4.25 - 7),  $W = 3.0$ ,  $p = 0.28$ , LQ diet first clutches: 5 (5 - 6), second clutches: 5 (4.5 - 6),  $W = 20.0$ ,  $p = 0.35$ ).

### **Sex ratios**

#### *Pooled data*

The number of male and female chicks produced on each diet in each round of breeding is summarised in Table 1. When the data were pooled for each diet from both rounds of

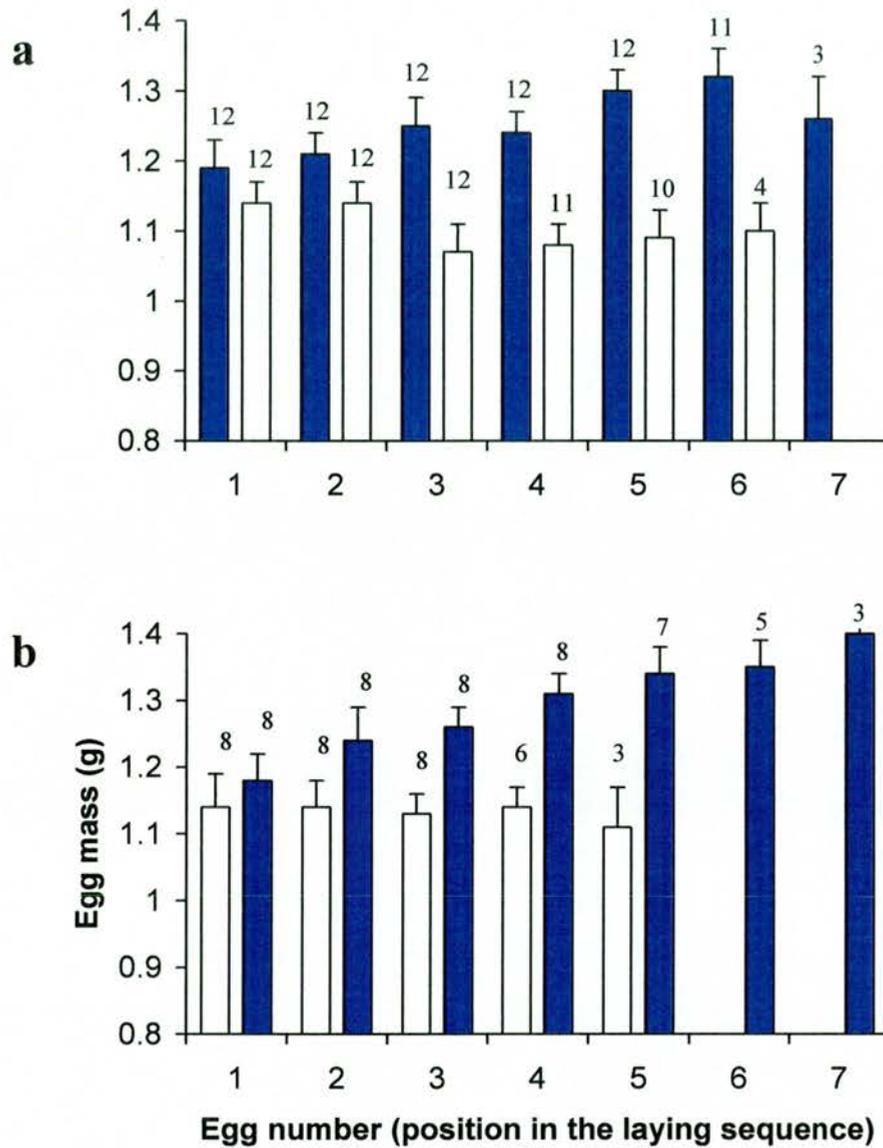
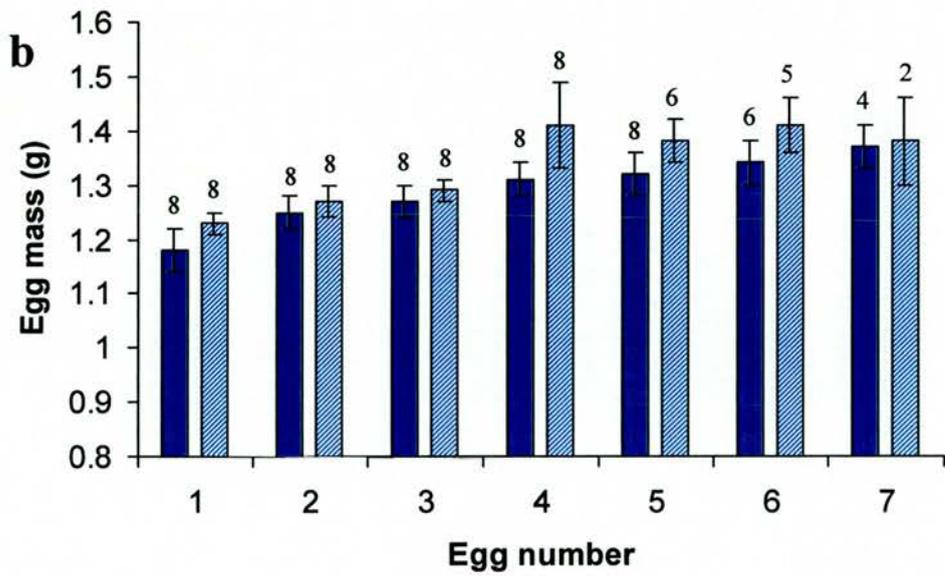
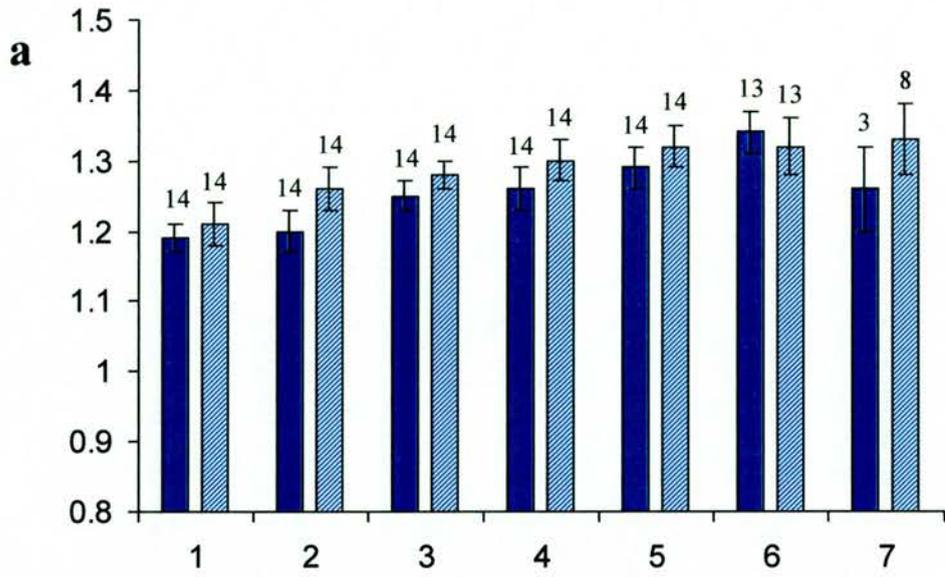


Figure 2. Mean mass of eggs ( $\pm$  SE) in relation to position in the laying sequence. Data are paired for females that laid (a) on the HQ diet in round one and on the LQ diet in round two ( $n = 12$ ) and (b) on the LQ diet in round one and on the HQ diet in round two ( $n = 8$ ). The blue bars represent the HQ diet eggs and the white bars represent the LQ diet eggs. Numbers of eggs are given above bars.

Figure 3 (overleaf). Mean egg mass ( $\pm$  SE) at each position in the laying sequence for first (filled bars) and second (hatched bars) clutches laid by (a) HQ diet females in round one (n = 14 females), (b) HQ diet females in round two (n = 8) and (c) LQ diet females in round two (n = 9). Numbers of eggs are given above bars.



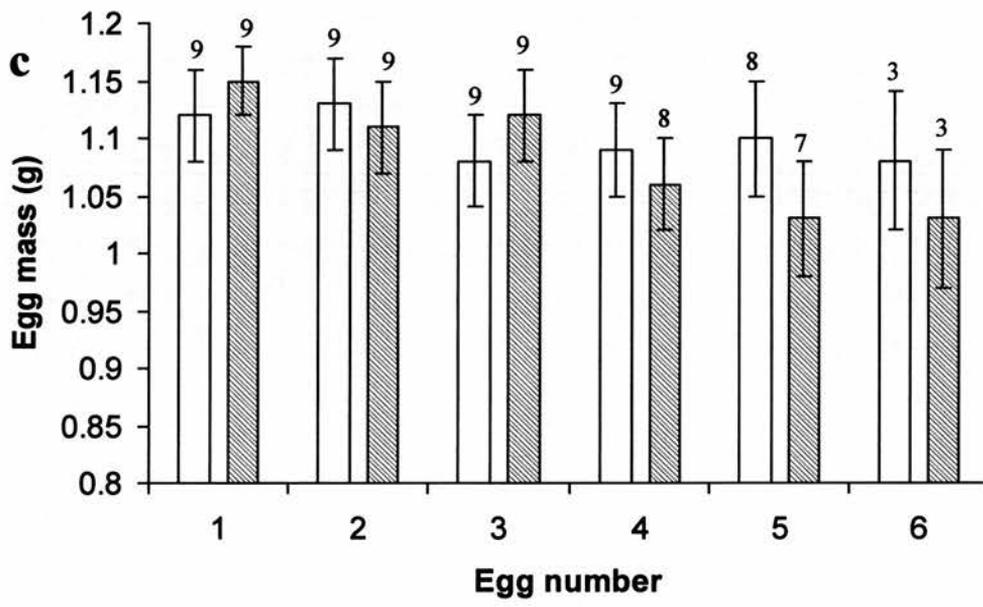


Table 1. The number of chicks of each sex at laying, hatching and fledging for females that were on the HQ diet and LQ diet in (a) round one and (b) round two. Sex ratio = males / (males + females). All females in round two had also laid clutches in round one. The numbers in brackets include second clutches.  $G_{adj1}$  values are goodness of fit against the Mendelian expectation of equal numbers of both sexes (\*  $p < 0.05$ ). M = males, F = females.

|          | Laying     |               |             |               | Hatching   |       |           |            | Fledging   |       |           |            |
|----------|------------|---------------|-------------|---------------|------------|-------|-----------|------------|------------|-------|-----------|------------|
|          | No. broods | M/F           | Sex ratio   | $G_{adj1}$    | No. broods | M/F   | Sex ratio | $G_{adj1}$ | No. broods | M/F   | Sex ratio | $G_{adj1}$ |
| <b>a</b> |            |               |             |               |            |       |           |            |            |       |           |            |
| HQ diet  | 14 (26)    | 36/47 (69/92) | 0.43 (0.42) | 1.45 (3.29)   | 14         | 30/42 | 0.42      | 2.00       | 14         | 23/38 | 0.38      | 3.70       |
| LQ diet  | 5          | 7/6           | 0.54        | 0.07          | 4          | 4/4   | 0.50      | 0          | 4          | 4/4   | 0.50      | 0          |
| <b>b</b> |            |               |             |               |            |       |           |            |            |       |           |            |
| HQ diet  | 7 (12)     | 16/19 (32/27) | 0.46 (0.54) | 0.25 (0.42)   | 7          | 11/15 | 0.42      | 0.61       | 5          | 9/9   | 0.50      | 0          |
| LQ diet  | 10 (20)    | 33/17 (54/35) | 0.66 (0.61) | 5.16* (4.06*) | 9          | 24/11 | 0.69      | 4.88*      | 7          | 15/6  | 0.71      | 3.89*      |

breeding, the sex ratio at laying (proportion of males) of 220 eggs laid from 38 clutches (first and second clutches combined) on the HQ diet was 0.46 ( $G_{adj 1} = 1.47$ ,  $p > 0.2$ ). On the LQ diet the sex ratio at laying of 102 eggs from 25 clutches (first and second clutches combined) was 0.60 ( $G_{adj 1} = 3.93$ ,  $p < 0.05$ ). At hatching the sex ratio on the HQ diet of 98 chicks from 21 broods was 0.42 ( $G_{adj 1} = 2.61$ ,  $p > 0.2$ ), whereas the sex ratio on the LQ diet of 43 chicks from 13 broods was 0.65 ( $G_{adj 1} = 3.95$ ,  $p < 0.05$ ). At fledging, the sex ratio on the HQ diet of 79 chicks from 19 broods was 0.41 ( $G_{adj 1} = 2.85$ ,  $p > 0.05$ ), whereas the sex ratio on the LQ diet of 29 chicks from 11 broods was 0.66 ( $G_{adj 1} = 2.79$ ,  $p > 0.05$ ).

#### *Paired data*

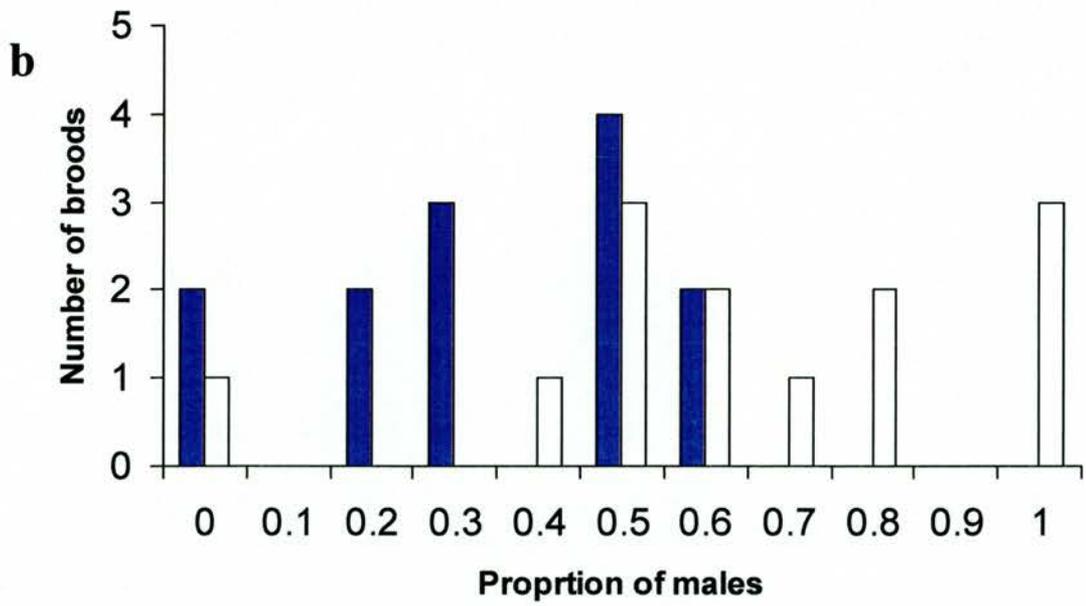
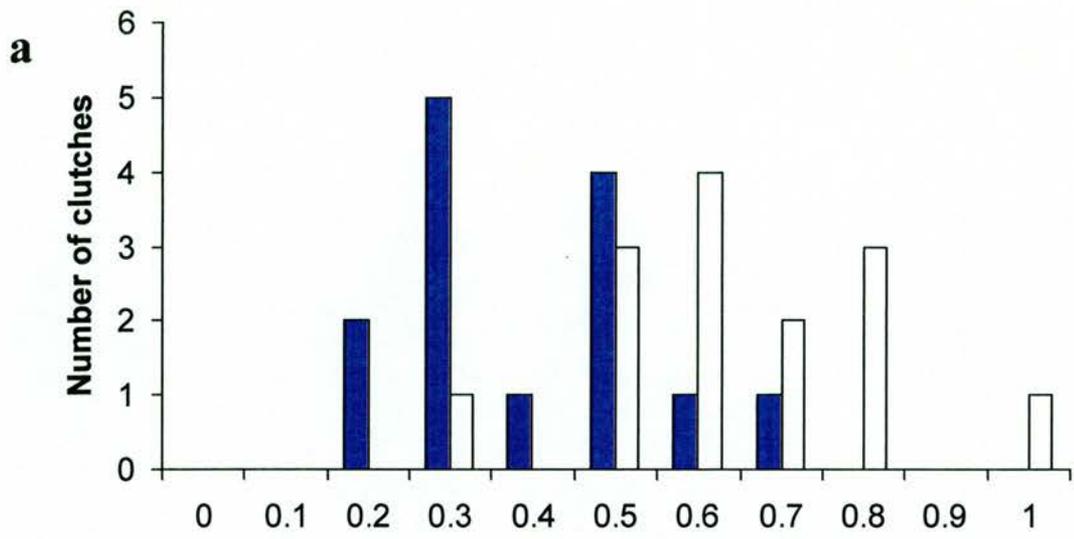
##### *Sex ratio at laying*

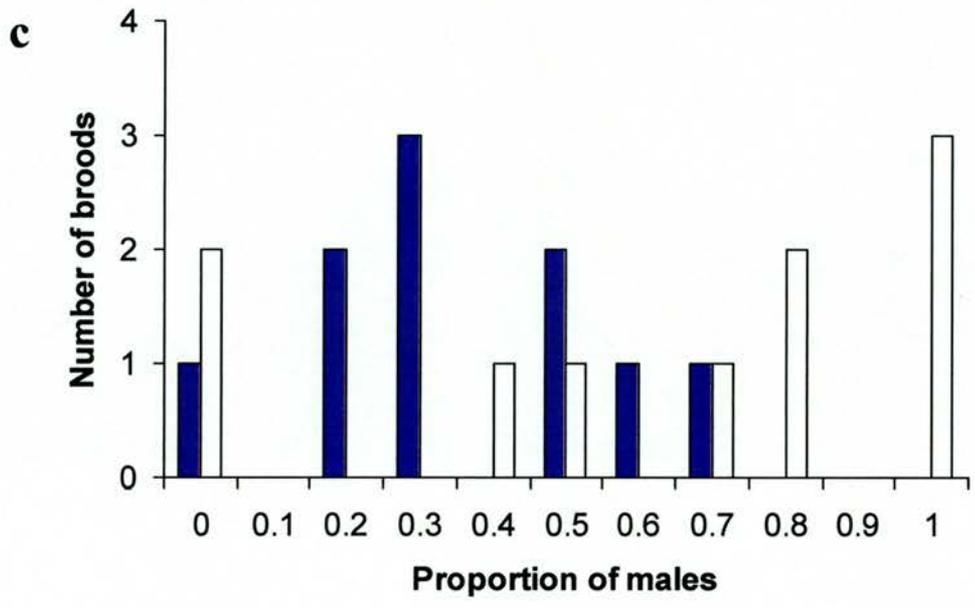
Paired analyses on the clutch sex ratio at laying (number of male eggs / total number of sexed eggs per brood) were carried out for 14 females that laid fertile eggs on both diets (10 females laid on the HQ diet and four on the LQ diet in round one). The clutch sex ratio was significantly more male-biased on the LQ diet than on the HQ diet (mean clutch sex ratio  $\pm$  SE, HQ diet:  $0.39 \pm 0.04$ , LQ diet:  $0.63 \pm 0.05$ ,  $F_{1,13} = 14.51$ ,  $p = 0.002$ , Fig. 4a) and there was no effect of order of treatment ( $F_{1,12} = 1.46$ ,  $p = 0.25$ ). The analysis of brood sex ratio was then conducted including second clutch eggs for females that laid on the HQ diet in round one and the LQ diet in round two (second clutches were not laid by females that were on the LQ diet in round one). The mean clutch sex ratio at laying remained significantly more male-biased on the LQ diet (HQ diet:  $0.41 \pm 0.04$ , LQ diet:  $0.66 \pm 0.04$ ,  $F_{1,19} = 16.69$ ,  $p = 0.0006$ ). There was no difference between the mean clutch sex ratio of first and second clutches ( $F_{1,18} = 0.04$ ,  $p = 0.85$ ).

##### *Sex and laying sequence*

Egg sex was analysed in relation to position in the laying sequence and experimental treatment. There was a significant interaction between diet and position in the laying sequence ( $F_{1,114} = 6.42$ ,  $p = 0.01$ , Fig. 5). The sex ratio decreased with position in the laying sequence on the HQ diet, i.e. became progressively more female-biased ( $F_{1,68} = 6.43$ ,  $p = 0.01$ ), and increased with position in the laying sequence on the LQ diet,

Figure 4 (overleaf). Frequency distribution of brood sex ratios (a) at laying (n = 14 clutches), (b) at hatching (n = 13 broods) and (c) at fledging (n = 10 broods) for females that bred on both diet treatments. Blue bars represent broods/clutches produced on the HQ diet and white bars represent broods/clutches produced on the LQ diet.





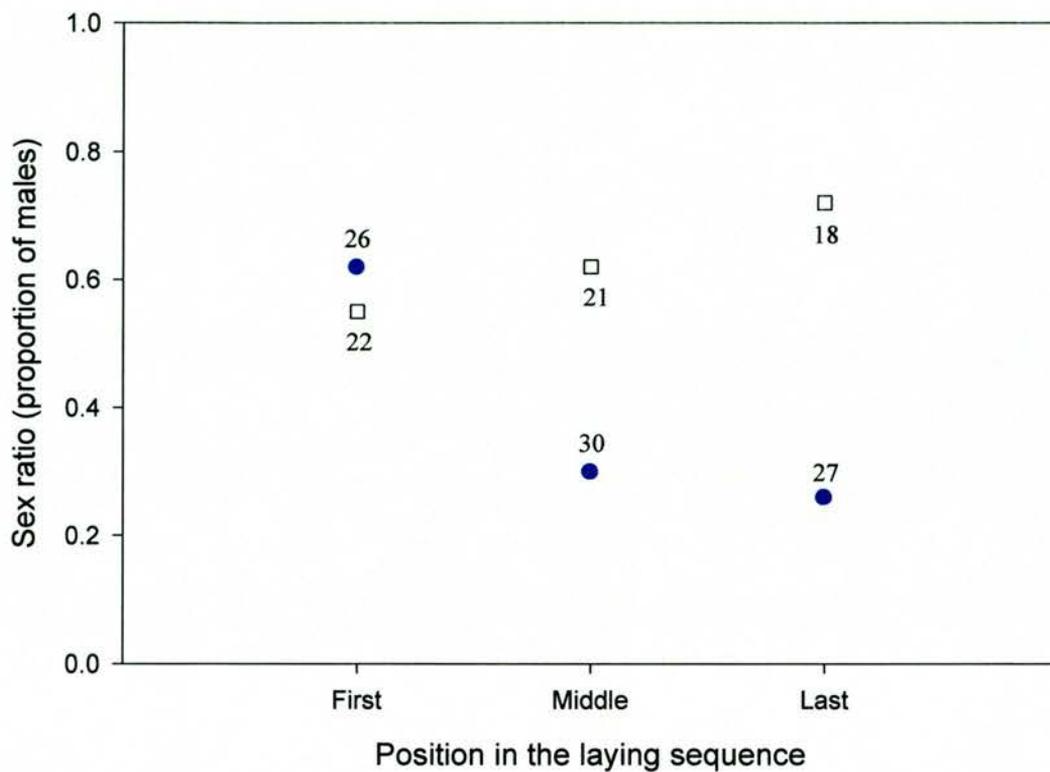


Figure 5. Sex ratio (proportion of males) at laying in relation to relative position in the clutch (first, middle or last) for first clutch eggs, for females that laid on both the HQ and LQ diets ( $n = 14$ ). Blue circles represent HQ diet eggs and white squares represent LQ diet eggs. Numbers beside points show the number of eggs that were sexed.

although not significantly so ( $F_{1,46} = 1.25$ ,  $p = 0.27$ ). When first and second clutches were combined for females that laid on the HQ diet in round one and the LQ diet in round two, the interaction between diet and laying sequence was still significant ( $F_{1,191} = 5.77$ ,  $p = 0.02$ ).

#### *Sex ratio at hatching*

At hatching, paired data were analysed for 13 females (nine females laid on the HQ diet and four females laid on the LQ diet in round one). Again, the brood sex ratio was significantly more male-biased on the LQ diet (mean brood sex ratio  $\pm$  SE, HQ diet:  $0.34 \pm 0.06$ , LQ diet:  $0.64 \pm 0.08$ ,  $F_{1,12} = 11.50$ ,  $p = 0.005$ , Fig. 4b) and there was no effect of order of treatment ( $F_{1,11} = 1.52$ ,  $p = 0.24$ ).

#### *Sex ratio at fledging*

At fledging, paired data were analysed for ten females (seven females laid on the HQ diet and three females laid on the LQ diet in round one). The brood sex ratio remained significantly different on the two diets (mean brood sex ratio  $\pm$  SE, HQ diet:  $0.35 \pm 0.07$ , LQ diet:  $0.61 \pm 0.12$ ,  $F_{1,9} = 5.84$ ,  $p = 0.04$ , Fig. 4c) and there was no effect of order of treatment ( $F_{1,8} = 1.32$ ,  $p = 0.28$ ).

### **Mortality**

#### *Embryo mortality*

Paired embryo mortality data were analysed in relation to diet, embryo sex, position in the laying sequence, breeding round and egg mass for the 14 females that laid fertile clutches on both diets. There was a significant interaction between diet and sex ( $F_{1,115} = 4.03$ ,  $p = 0.047$ ). This was because, on the HQ diet, male embryo mortality (18.2% (6/33)) tended to be higher than that of females (8% (4/50),  $F_{1,68} = 1.92$ ,  $p = 0.17$ ). On the LQ diet female embryo mortality (37.5% (9/24)) tended to be higher than that of males (26.3% (10/38),  $F_{1,47} = 2.32$ ,  $p = 0.13$ ). There was no effect of order of treatment on embryo mortality ( $F_{1,117} = 1.17$ ,  $p = 0.28$ ). There was no effect of laying sequence on embryo mortality ( $F_{1,113} = 0.15$ ,  $p = 0.70$ ) and no interaction between laying sequence and diet ( $F_{1,112} = 0.01$ ,  $p =$

0.93). Finally, there was no effect of egg mass on embryo mortality on either diet ( $F_{1,114} = 0.71$ ,  $p = 0.40$ ).

#### *Post-hatching mortality*

Paired data on post-hatching mortality was analysed in relation to diet, chick sex, breeding round, position in the laying sequence, egg mass and brood size at hatching. There was no difference in mortality between male and female chicks ( $F_{1,82} = 0.18$ ,  $p = 0.67$ ), and the interaction between diet and sex was not significant ( $F_{1,81} = 0.74$ ,  $p = 0.39$ ). The single significant predictor of post-hatching mortality was egg mass ( $F_{1,83} = 17.53$ ,  $p < 0.0001$ , Fig. 6). There was no effect of diet after controlling for egg mass ( $F_{1,83} = 1.93$ ,  $p = 0.17$ ). Chicks that died before fledging came from significantly lighter eggs both on the HQ diet ( $F_{1,52} = 8.19$ ,  $p = 0.006$ ) and on the LQ diet ( $F_{1,30} = 9.39$ ,  $p = 0.005$ ).

### **Chick development**

#### *Hatchlings*

Paired data were analysed for 13 pairs that hatched young on both the HQ and LQ diets (nine females were on the HQ diet and four females on the LQ diet in round one, Table 2). Hatching mass was strongly related to egg mass, but hatching mass was also affected by order of treatment (Fig. 7a). For females that were on the HQ diet in round one, chicks hatched at significantly greater mass than when on the LQ diet ( $F_{1,34} = 8.34$ ,  $p = 0.007$ ). However, there was no difference in hatching mass between chicks produced by females that were on the LQ diet in round one and on the HQ diet in round two ( $F_{1,4} = 0.04$ ,  $p = 0.85$ ). The same was true for hatchling tarsus length (Table 2, Fig. 7b). Hatchling tarsus length was significantly longer for chicks from females that were on the HQ diet in round one compared to chicks hatched by these females that were on the LQ diet in round two ( $F_{1,33} = 216.98$ ,  $p < 0.0001$ ). However, there was no significant difference between chicks from females that were on the LQ diet in round one and those from these females on the HQ diet in round two ( $F_{1,4} = 0.00$ ,  $p = 0.97$ ). There was no relationship between hatchling tarsus length and egg mass ( $F_{1,51} = 0.02$ ,  $p = 0.88$ ). There was no effect of chick sex on hatchling tarsus length ( $F_{1,99} = 0.29$ ,  $p = 0.59$ ) and no interaction between diet and chick sex ( $F_{1,92} = 0.86$ ,  $p = 0.36$ ). Similarly, mass at hatching did not differ between male and

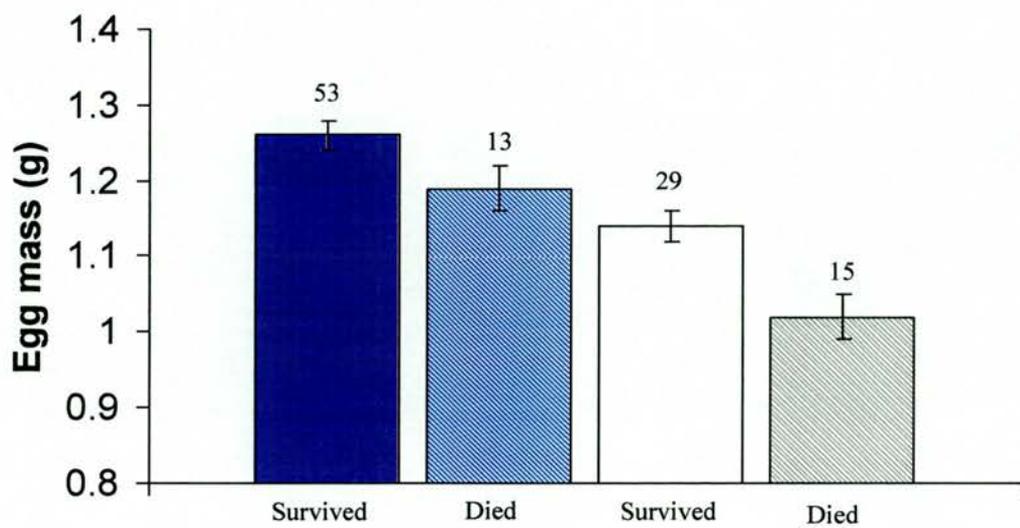


Figure 6. Mass of eggs (mean  $\pm$  SE) from which chicks hatched that survived to fledging (filled bars), or died after hatching (hatched bars) on the HQ and LQ diets. Blue bars represent HQ diet eggs and white bars represent LQ diet eggs. Number of eggs are given above bars.

Table 2. Factors influencing the mass and tarsus length of hatchlings (GLM analyses). Data are paired for 13 females that hatched young on both diets. Non-significant terms were removed from the model and hence not shown.

| <b>Variable</b>               | <b>df</b> | <b>F</b> | <b>p</b> |
|-------------------------------|-----------|----------|----------|
| <i>Hatching mass</i>          |           |          |          |
| <b>Egg mass</b>               | 1,52      | 143.55   | < 0.0001 |
| <b>Diet</b>                   | 1,63      | 3.17     | 0.08     |
| <b>Breeding round</b>         | 1,62      | 0.28     | 0.60     |
| <b>Diet * breeding round</b>  | 1,36      | 7.53     | 0.009    |
| <i>Hatching tarsus length</i> |           |          |          |
| <b>Diet</b>                   | 1,59      | 43.96    | < 0.0001 |
| <b>Breeding round</b>         | 1,59      | 47.90    | < 0.0001 |
| <b>Diet * breeding round</b>  | 1,35      | 6.22     | 0.02     |

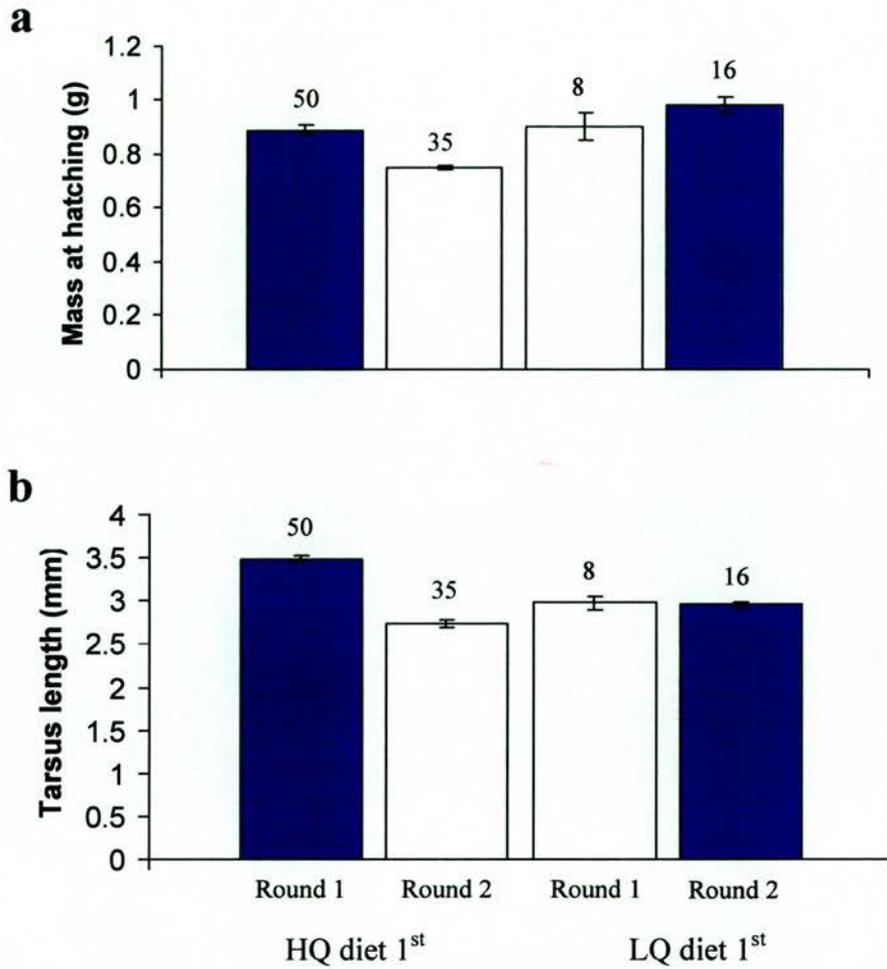


Figure 7. Paired (a) mass and (b) tarsus length (mean  $\pm$  SE) for hatchlings from females that hatched young on both diets (nine females were on the HQ diet and four females were on the LQ diet in round one). Blue bars represent the HQ diet treatment and white bars represent the LQ diet treatment. Numbers of chicks are given above bars.

female chicks ( $F_{1,101} = 0.40$ ,  $p = 0.53$ ) and there was no interaction between diet and sex ( $F_{1,95} = 0.66$ ,  $p = 0.42$ ).

### *Fledglings*

Paired data were analysed for ten pairs that fledged young on both the HQ and LQ diets (seven females bred on the HQ diet and three females bred on the LQ diet in round one, Table 3). The brood size at fledging was significantly greater on the HQ diet (mean  $\pm$  SE,  $4.21 \pm 0.64$ ) compared with on the LQ diet ( $1.93 \pm 0.40$ , paired t-test,  $t = 3.39$ ,  $p = 0.005$ ,  $n = 10$ ).

Regardless of order of treatment, offspring reared on the HQ diet fledged earlier and at heavier mass than offspring reared on the LQ diet (Fig. 8). Offspring reared on the HQ diet in round one fledged at greater mass than offspring reared by these pairs on the LQ diet in round two ( $F_{1,10} = 7.02$ ,  $p = 0.02$ ) and at a younger age ( $F_{1,12} = 37.73$ ,  $p < 0.0001$ ). The same was true for offspring that fledged on the LQ diet in round one and the HQ diet in round two (difference in mass:  $F_{1,10} = 26.18$ ,  $p = 0.0005$ , difference in age:  $F_{1,4} = 44.28$ ,  $p = 0.004$ ). On the LQ diet fledging mass decreased with position in the laying sequence ( $F_{1,10} = 8.37$ ,  $p = 0.02$ ), but not on the HQ diet ( $F_{1,12} = 0.46$ ,  $p = 0.51$ ). Similarly, tarsus length at fledging decreased with position in the laying sequence for LQ diet offspring ( $F_{1,10} = 5.24$ ,  $p = 0.04$ ) but not for HQ diet offspring ( $F_{1,20} = 2.20$ ,  $p = 0.15$ ).

### *Adults*

Paired data on mass and tarsus length when offspring reached adulthood (120 days) were analysed for ten pairs. HQ diet offspring tended to be heavier than LQ diet offspring ( $F_{1,18} = 4.31$ ,  $p = 0.052$ ) and had significantly longer tarsi ( $F_{1,16} = 5.52$ ,  $p = 0.03$ ). The interaction between diet and breeding round was not significant for either adult mass or tarsus length ( $p > 0.1$  in both cases). On both diets females were significantly heavier than males ( $F_{1,52} = 8.43$ ,  $p = 0.005$ , Fig. 9). There were no differences in tarsus length between males and females ( $F_{1,58} = 0.00$ ,  $p = 0.99$ ) and the interaction between diet and sex with respect to tarsus length was not significant ( $F_{1,55} = 0.00$ ,  $p = 0.96$ ).

Table 3. Factors influencing the age, mass and tarsus length of fledglings (GLM analyses). Data are paired for 10 females that fledged young on both diets. Non-significant terms were removed from the model and hence not shown.

| <b>Variable</b>                | <b>df</b> | <b>F</b> | <b>p</b> |
|--------------------------------|-----------|----------|----------|
| <i>Age at fledging</i>         |           |          |          |
| <b>Diet</b>                    | 1,13      | 30.20    | 0.0001   |
| <b>Breeding round</b>          | 1,13      | 1.05     | 0.32     |
| <b>Laying sequence</b>         | 1,20      | 7.88     | 0.01     |
| <b>Diet * breeding round</b>   | 1,36      | 7.53     | 0.009    |
| <i>Fledgling mass</i>          |           |          |          |
| <b>Diet</b>                    | 1,44      | 0.88     | 0.35     |
| <b>Breeding round</b>          | 1,14      | 0.47     | 0.50     |
| <b>Laying sequence</b>         | 1,47      | 12.29    | 0.0001   |
| <b>Diet * breeding round</b>   | 1,16      | 19.23    | 0.0005   |
| <b>Diet * laying sequence</b>  | 1,34      | 5.41     | 0.03     |
| <i>Fledgling tarsus length</i> |           |          |          |
| <b>Diet</b>                    | 1,43      | 1.39     | 0.24     |
| <b>Laying sequence</b>         | 1,45      | 0.19     | 0.66     |
| <b>Diet * laying sequence</b>  | 1,30      | 4.21     | 0.049    |

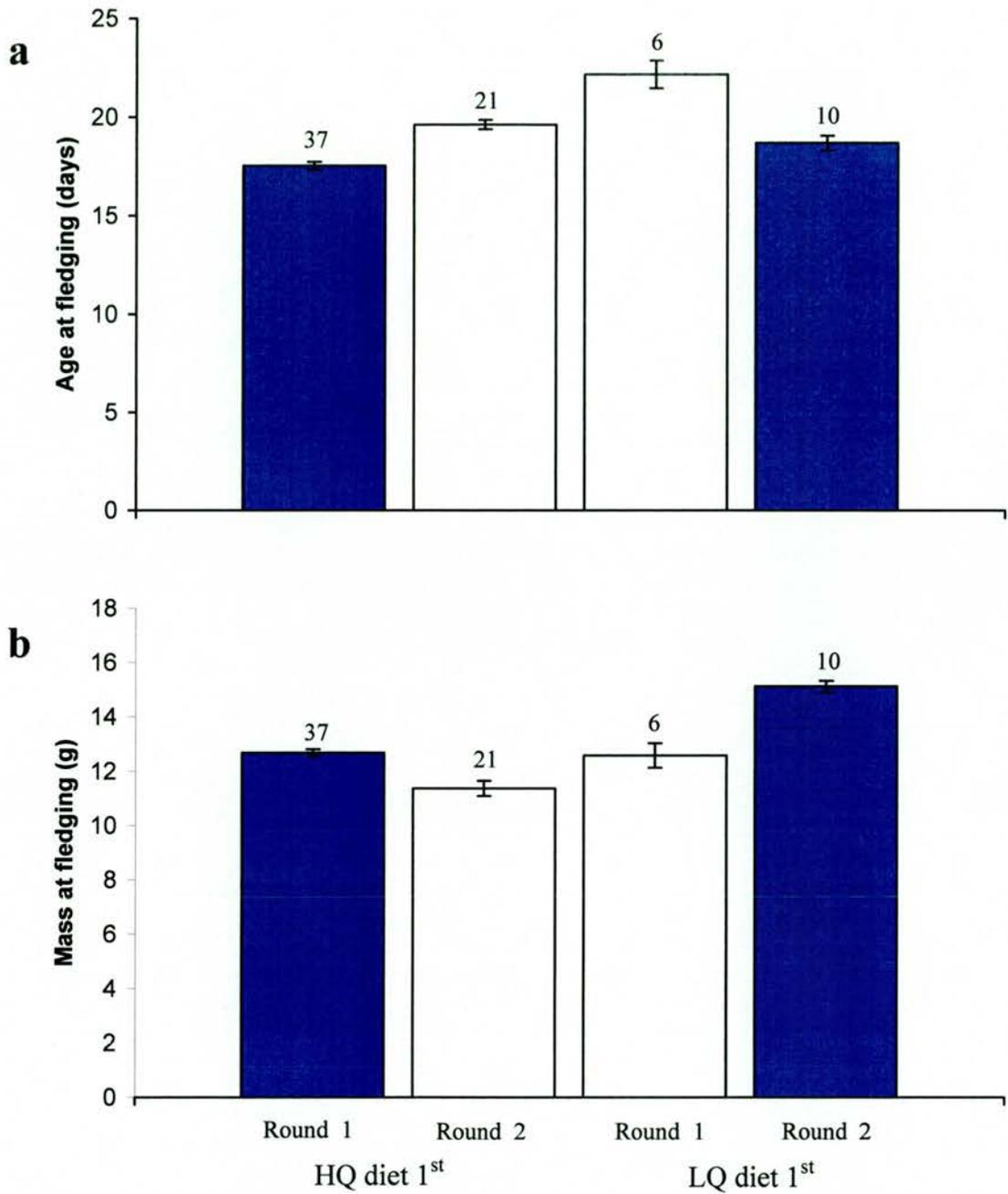


Figure 8. Paired (a) age at fledging and (b) mass at fledging (mean  $\pm$  SE) for chicks from females that fledged young on both diets (seven females were on the HQ diet and three were on the LQ diet in round one). Blue bars represent the HQ diet treatment and white bars represent the LQ diet treatment. Numbers of chicks are given above bars.

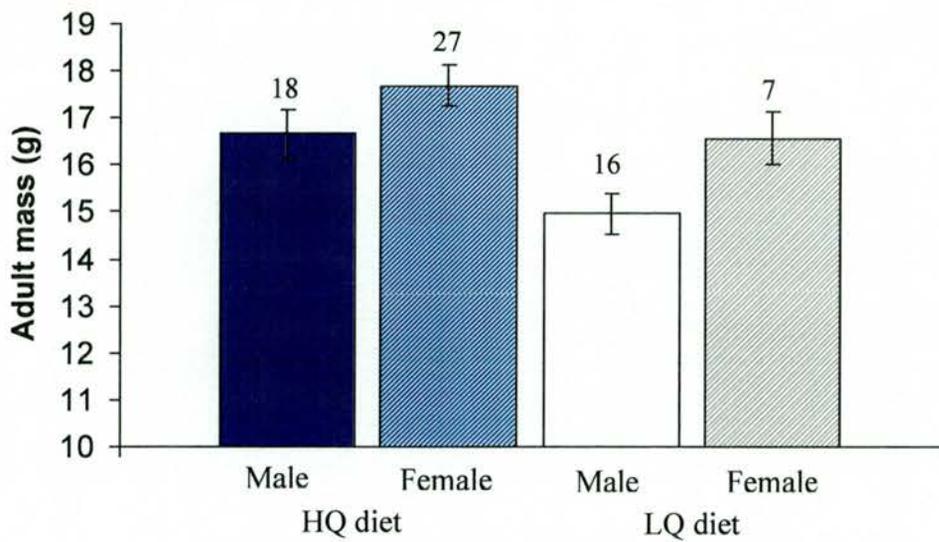


Figure 9. The mass (mean  $\pm$  SE) of male and female offspring upon reaching adulthood (120 days) reared on the HQ and LQ diets. Data presented are of offspring from females that reared chicks to adulthood on both diets (n = 9 females). Data are pooled for both rounds of breeding. Numbers of chicks are given above bars.

### **Mass change of females**

Paired analyses were carried out on the 20 females that laid clutches on both diets. Experimental diet had no effect on female mass during the period for which they were on the diet prior to pairing ( $F_{1,19} = 20.7$ ,  $p = 0.12$ ) and there was no effect of order of treatment ( $F_{1,18} = 2.69$ ,  $p = 0.12$ ). Paired analyses on mass change during breeding revealed an almost significant three-way interaction between diet, breeding round and whether or not they reared a chick to fledging ( $F_{1,30} = 3.60$ ,  $p = 0.064$ , Fig. 10a). However, for females that were on the HQ diet in round one ( $n = 12$ ), mass change during breeding was neither significantly affected by diet ( $F_{1,11} = 3.88$ ,  $p = 0.07$ ), nor by whether or not they reared chicks ( $F_{1,20} = 1.17$ ,  $p = 0.29$ ) on either diet. The interaction between diet and whether or not they reared chicks was not significant ( $F_{1,13} = 1.42$ ,  $p = 0.26$ ). For females that were on the LQ diet in round one ( $n = 8$ ), mass loss was greater during breeding on the LQ diet ( $F_{1,7} = 20.02$ ,  $p = 0.003$ ) and females lost more mass when they reared chicks ( $F_{1,8} = 9.67$ ,  $p = 0.01$ ). Again, the interaction between diet and whether or not they reared chicks was not significant ( $F_{1,7} = 3.21$ ,  $p = 0.12$ ).

### **Mass change of males**

Paired analyses on mass change during breeding were carried out on 20 males, whose mate had laid a clutch on both diets (Fig. 10b). There was a significant interaction between diet and breeding round ( $F_{1,20} = 27.75$ ,  $p < 0.0001$ ) since males that received the LQ diet in round one lost more mass than males that received the HQ diet in round one. There was also a significant interaction between breeding round and whether or not they reared chicks ( $F_{1,25} = 5.03$ ,  $p = 0.03$ ). In round one, males that reared chicks lost more mass than in round two (Fig. 10b).

## **Discussion**

### **Reproductive success**

Diet quality had a very strong effect on reproductive success, with females rearing, on average, twice the number of chicks to independence on the HQ diet. There were no differences in the proportion of females that laid eggs in the two treatments or, in the number of days between pairing and laying. However, females laid significantly larger

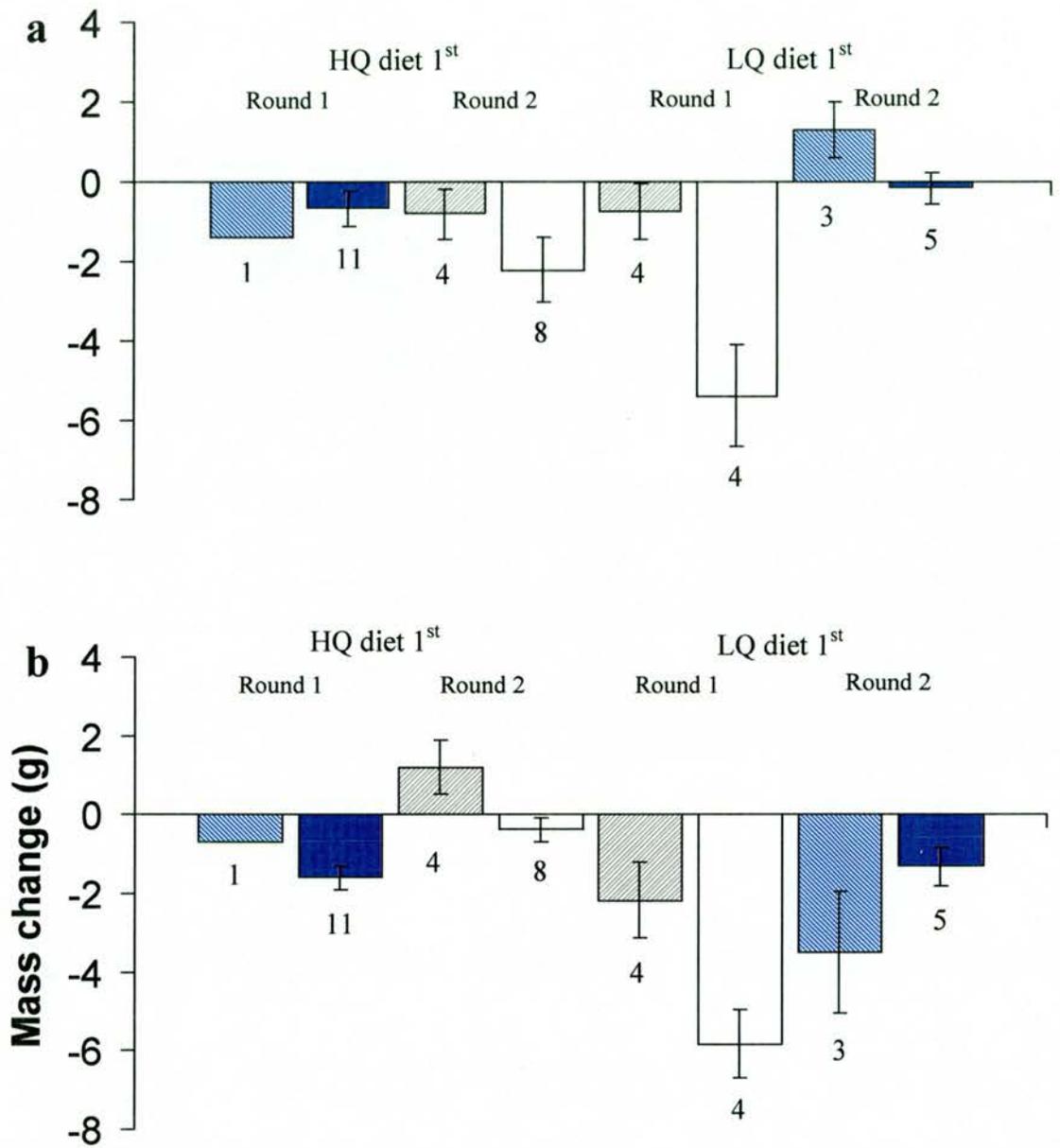


Figure 10. Mass change (means  $\pm$  SE) during breeding for (a) females that laid clutches on both diets and for (b) males whose mate laid a clutch on both diets. The left half of the graph represents birds that were on the HQ diet in round one and the LQ diet in round two ( $n = 12$ ). The right half of the graph represents females that were on the LQ diet in round one and the HQ diet in round two ( $n = 8$ ). Hatched bars are birds that did not rear chicks to fledging in that treatment, filled bars are birds that did rear at least one chick to fledging.

clutches and heavier eggs when they bred on the HQ diet compared with on the LQ diet. Since females are thought to be protein-limited during egg formation (Houston *et al.* 1995, Williams 1996a), such a result is unsurprising, and has been previously reported in other zebra finch studies (Monaghan *et al.* 1996, Selman & Houston 1996, Williams 1996a, Rutkowska & Cichon 2002). The pattern of smaller clutch and egg size when on the LQ diet could also result from maternal differential allocation; when resources are limited, reproduction is more costly in terms of the female's body condition and future reproductive potential. In addition, fewer offspring can be reared (Perrins 1970), and these offspring will be of lower reproductive value (Hussell 1972, Hochachka 1992).

### **Egg mass and laying sequence**

The pattern of intra-clutch egg mass differed on the two diets. On the HQ diet, egg mass increased with position in the laying sequence, whereas on the LQ diet, egg mass tended to decrease with position in the laying sequence. A decline in egg mass with laying sequence might reflect a brood reduction strategy (Parsons 1970, O'Connor 1979, Lundberg & Vaisainen 1979) since hatching at smaller mass further increases the disadvantage of hatching last. However, a decline in egg mass with laying sequence might represent physiological constraints on these females due to depletion of resources (Pierotti & Bellrose 1986, Sydeman & Emslie 1992). In contrast, an increase of egg mass with laying sequence could reflect a brood survival strategy (Howe 1976, Clark & Wilson 1981, Slagsvold *et al.* 1984), whereby females are investing more in later eggs to offset the disadvantage to these chicks of hatching last and having to compete with older, larger siblings. A pronounced increase in egg mass with laying sequence was also found in a zebra finch study by Rutkowska & Cichon (2002), when diet quality was increased after the first egg was laid, possibly reflecting food availability during egg formation. However, in the current study, diet quality was constant during the egg laying period and for a long time prior to laying, making an adaptive explanation for an increase in egg mass with laying sequence seem likely.

In the current study, the degree of hatching asynchrony was highly variable with gaps of up to a week between the oldest and youngest chick, particularly in larger broods (pers. obs.). Hatching is reported to be more synchronous in the wild (Zann 1996), and so

may have been an artefact of the laboratory situation although, hatching asynchrony has also been reported in second-generation wild-caught birds (D. Gil, pers. comm.). Since hatching mass correlates with egg mass, hatching mass increased with hatching sequence (which corresponded with laying sequence) only in the HQ group. Post-hatching mortality was higher for chicks that hatched from lighter eggs in both groups. Therefore, greater hatching mass of late-hatching chicks on the HQ diet would be important in determining survival, especially in large broods. Furthermore, at fledging, mass and tarsus length decreased with laying sequence only on the LQ diet but not on the HQ diet. Differences in egg mass and laying sequence have also been reported in relation to maternal age in red-winged blackbirds (Blank & Nolan 1983). In this species, the energy content of eggs of old mothers increased with laying sequence, but did not increase in middle-aged and young females. This resulted in higher starvation rates among chicks that hatched from later eggs in younger females, but not older females.

### **Hatching success**

In the first round of breeding, the LQ group females had very poor reproductive success, with only eggs from four clutches hatching. The remainder of the clutches were abandoned early on or infertile. Wild zebra finches are adapted to breed on even a minimal diet (Zann 1996), but this is not the case with laboratory finches. Low hatching success on a LQ diet has been found in other zebra finch studies (Selman & Houston 1996, Gorman & Nager 2003). Kilner (1998) did not, however, find a difference in hatching success as a result of differences in food abundance (she also did not find a difference in clutch size). All 12 females that reared young in the round one on the HQ diet also reared young when on the LQ diet in round two. These females had already bred with their mate and this might have been part of the reason for their relative success compared with LQ first round breeding birds (Black 2001). Alternatively, or additionally, there could have been a carry-over effect from the earlier, HQ diet (see also Chapter 5). Williams (1996a) found that the effects of a supplementary diet on egg mass during egg laying lasted for a considerable time after the diet was stopped. He suggested that females might store some limiting essential amino acids that were then used over a longer period than that coinciding with immediate dietary intake.

## Offspring development

### *At hatching*

Mass at hatching correlated strongly with egg mass and so there was a large difference between the two diets. There was also a strong effect of order of treatment. There was a larger difference between the mass of hatchlings from females that were on the HQ diet in round one and the LQ diet in round two, compared with chicks from females that were on the LQ diet in round one and the HQ diet in round two. This may be because females that were on the LQ diet in round two were more physiologically stressed, since they reared larger broods and laid an extra clutch of eggs in round one compared with females on the LQ diet in round one. Relatively few females hatched young on the LQ diet in round one, and those that did reared smaller broods. The data for hatchling tarsus length showed a similar pattern. Therefore the eggs may have differed in quality depending on the order of treatment, since egg mass was not affected by order of treatment. There was no sex difference in mass or tarsus length at hatching, although a previous zebra finch study found that males were heavier at hatch (Rutkowska & Cichon 2002).

### *At fledging*

Chicks reared on the HQ diet fledged earlier and at greater mass. This was true for both orders of treatment, unlike the hatchling data. Rapid maturation is advantageous in this precocial-breeding species (Zann 1994a) and fledging mass has important fitness consequences, since in many species it correlates strongly with survival in the wild and recruitment into the breeding population (Perrins 1965, Garnett 1981, Magrath 1991). Since there was no cross-fostering of eggs or chicks in this experiment, these differences could have been primarily attributable to differences in egg quality, parental quality or rearing diet. Egg quality is probably of greater importance early on in the nestling's development (Amundsen & Stockland 1990, Williams 1994), and parental care is probably more important in species with a relatively long nestling period (Bolton 1991). Egg size may be particularly important in harsh conditions (Smith *et al.* 1995, Smith & Bruun 1998) since, in favourable conditions extended parental care might compensate for small eggs and enable small hatchlings to catch up (Styrsky *et al.* 1999).

### *At adulthood*

As adults offspring on the HQ diet were heavier and had longer tarsi than offspring on the LQ diet. Other studies on the effects of rearing diet on zebra finch development have found evidence for catch-up or compensatory growth on either adult mass, tarsus length, or both, as well as other morphological traits such as beak colour (Skagen 1988, de Kogel 1997, Birkhead *et al.* 1999, but not Boag 1987). This would probably be the case in the current experiment after a longer time interval. There is evidence that compensatory growth can adversely affect the immune system and longevity (Lochmiller *et al.* 1993, Birkhead *et al.* 1999), but this may be an acceptable trade-off in short-lived species such as the zebra finch (Metcalf & Monaghan 2001).

Females were heavier as adults than males. This was predicted on the HQ diet since high quality daughters should have higher fitness returns than high quality males (Kilner 1998), but the converse was predicted on the LQ diet, since females are more vulnerable to nutritional stress than males (de Kogel 1997, Bradbury & Blakey 1998, Kilner 1998, Martins 2003). However, there were relatively few females on the LQ diet, and it is possible therefore that poor quality females did not reach adulthood.

### **Second clutches**

Females that laid second clutches on the HQ diet in both rounds of breeding laid heavier eggs. It has been shown previously (Williams 1996a, 1996b) that females lose less mass after laying second compared with first clutches, suggesting some physiological differences between clutches, which might explain why females laid heavier eggs. A second possibility is that females were investing more in their second clutch because they had already successfully reared a clutch with their mate and were prepared to invest more the second time.

In contrast, females on the LQ diet in round one did not lay second clutches at all in the timescale of this round of breeding. In round two, females on the LQ diet laid second clutches, but these were not heavier than first clutches. This is probably because these latter females were more nutritionally constrained after having reared chicks on the LQ diet and had lost a substantial amount of body mass.

### **Sex allocation**

There was no difference in the mass of male and female eggs, which was also the case in the study by Rutkowska & Cichon (2002). However, as predicted, the sex ratio at laying was significantly different on the two diets. Females laid a significantly higher proportion of male eggs on the LQ diet, and a higher proportion (although this was not statistically significant) of female eggs on the HQ diet. A male bias in poor dietary conditions was also found by Kilner (1998) and Bradbury & Blakey (1998) although, they only sexed chicks at hatching and not at laying, by which time there may have already have been substantial mortality (23% of eggs failed to hatch in Kilner's experiment). Neither of these studies found a female-biased sex ratio on a good diet, and this was also true of a study on wild zebra finches (Zann & Runciman 2003).

Rutkowska & Cichon (2002) found that females whose diet deteriorated after the first egg had been laid, produced more male eggs, thus demonstrating their ability to adjust the sex ratio in relation to short term changes in food supply. They suggested that females were adjusting the sex ratio in response to resources available in the egg. This differs from the explanation given by Bradbury & Blakey (1998) who argued that females adjusted the sex ratio in relation to their own body condition. Bradbury & Blakey found that females on a HQ diet were in poorer condition, whereas females on a LQ diet were in good condition because they stored proteins necessary for egg production. They argued that males were the more costly sex (because of their slightly larger size) and so were produced by females in good condition. The results of the current study disagree with those of Bradbury & Blakey (1998), since females on a HQ diet were found to be in better condition (they lost less mass and laid heavier second clutches). Therefore, females could be adjusting the sex ratio in relation to their own body condition, the quality of the eggs, and the perceived future rearing environment.

### **Sex, laying sequence and mortality**

Although there were no differences in the mass of male and female eggs on the two diets, there was a difference in the relative position in the laying sequence of male and female eggs. Female eggs were laid earlier in the laying sequence on the LQ diet and the converse

was true on the HQ diet. Kilner (1998) found that females hatched earlier than males under both restricted and abundant food regimes and suggested that females were more sensitive than males and so should be in the favoured position. She did not report any changes in egg mass with laying sequence. In the current experiment, because egg mass increased with position in the laying sequence on the HQ diet, and because heavier eggs had lower post-hatching mortality, the favoured position on the HQ diet would be later in the laying sequence. On the LQ diet, biasing early eggs towards females might be adaptive since these eggs tended to be heavier and so would have higher survival prospects, although this contradicts the idea that they are adopting a brood reduction strategy. Rutkowska & Cichon (2002) suggested that female eggs were laid first in their study because there was higher male embryo mortality, and so males benefited more from being laid later on the laying sequence when egg mass increased with laying sequence. More work is needed to investigate differential embryo mortality in relation to egg quality. Higher male embryo mortality was found in Chapter 3 when females were mated with an attractive male, but strong differences in embryo mortality were not found in either diet in the current study. It is well established that females are more vulnerable on a poor diet post-hatching (de Kogel 1997, Bradbury & Blakey 1998, Kilner 1998, but not in the current study). However, this could reflect egg quality (Nager *et al.* 1999), rearing diet (Martins 2003) as well as differential parental provisioning (Clotfleter 1996) and sibling competition (Oddie 2000). In zebra finches, only rearing diet has been looked at in isolation so far (Martins 2003) and found that males gain mass faster than females on a restricted diet. The other possibilities need to be investigated as well to obtain the full picture.

### **Female condition**

Females on the HQ diet had greater reproductive success, and they were in better condition, since they lost less body mass during breeding, suggesting that they incurred lower reproductive costs than did LQ diet females. Similarly, Skagen (1988) found that limited food caused females to lose more weight than when food was not limited, and Williams (1996a) also found that protein-fed females did not lose body mass during egg laying compared with controls. Additionally, in the current study, females on the HQ diet laid heavier second clutches in both the first and second rounds of breeding, providing

further testament to their good condition (in the second round of breeding the LQ diet females did not lay heavier eggs in their second clutches and, in the case of females that were on the LQ diet first, did not lay second clutches at all), again suggesting that because they incurred lower costs, they had more reserves for subsequent reproduction (Reid *et al.* 2000). As mentioned earlier, this contradicts the findings of Bradbury & Blakey (1998). Their HQ diet females were in poorer condition as indicated by fat and muscle scores, their explanation for which was that the length of the pre-breeding diet (3 months), led to the females on the LQ diet laying down reserves, whereas the females on the HQ diet did not. One reason for the discrepancy might be that their diet was lower in protein content than in the current study. Selman & Houston (1996) found that HQ diet birds lost significantly less pectoral muscle tissue per gram of egg laid than LQ diet birds because they received the necessary amino acids for egg production prior to egg laying. HQ diet females consequently needed to break down smaller quantities of muscle protein. In their study females had to store these proteins since the HQ diet was ended prior to egg laying. In the current study, since the HQ diet was continued through egg laying and chick rearing, females would be expected to remain in even better condition, as they would continue to obtain the necessary protein from their diet.

### **Conclusions**

The current study demonstrated that female zebra finches show very different reproductive strategies on a LQ and HQ diet, with respect to egg mass and laying sequence, and differential sex allocation. Females produced more of the sex that has been shown to have the highest fitness returns under those conditions. In addition, although there was no difference in the mass of male and female eggs, female eggs were laid later in the laying sequence on the HQ diet and since these eggs were heavier, this would enhance their fitness. These adaptations probably reflect the unpredictable environment in which zebra finches have evolved, and in which they still reproduce in the wild (Hirshfield & Tinkle 1975).

## Chapter 4 Appendix: Diet quality pilot study

### Methods

#### Experimental design

Thirty female zebra finches were weighed and randomly assigned to one of two diet groups. Females on the HQ diet were given foreign finch mix *ad libitum*, supplemented with daily portions of Haith's egg biscuit and fresh spinach. Females on the LQ diet were given panicum millet *ad libitum* (12% protein). Both groups had access to cuttlefish bone and fresh water *ad libitum*.

Females were kept on these diets in single sex groups for a period of six weeks, after which they were re-weighed and paired randomly with males with whom they had not previously bred. The pairs were moved into individual breeding cages, equipped with open-design nest boxes and supplied with nesting material. For the six weeks prior to pairing, all males had been caged in a separate room, on the standard laboratory diet of foreign finch mix, supplemented with spinach and egg biscuit once a week. Once paired, males were then kept on the same diet as their mate, and these diets were maintained during chick rearing.

Nest boxes were checked for eggs and chicks as described in Chapter 2. Chicks were weighed as soon as possible after hatching to the nearest 0.01g. They were also weighed and measured (tarsus length) at fledging and at adulthood (120 days), as described in Chapter 2. At sexual maturity, chicks were sexed according to plumage characteristics. Chicks that died prior to sexual maturity, and eggs that were fertile but failed to hatch, were sexed using PCR, as described in Chapter 2.

### Results

#### Clutch size and egg mass

Within three weeks of pairing, 13/15 females laid eggs on the HQ diet and 11/15 females laid eggs on the LQ diet ( $G = 0.78$ ,  $df = 1$ ,  $p > 0.3$ ). Females on the HQ diet laid significantly larger clutches than females on the LQ diet, but mean egg mass per clutch was not significantly different, once female mass was controlled for in an ANCOVA (Table 1).

In a repeated measures GLM, female mass had a highly significant effect on egg mass in both diet treatments ( $F_{1,22} = 33.25$ ,  $p < 0.0001$ , Fig. 1). In addition, there

Table 1. Laying data for females on the HQ and LQ diets. Differences in days to laying and clutch size were analysed using a Mann-Whitney U test. The difference in the mean egg mass per clutch was analysed using an ANCOVA, with female body mass as a covariate.

|   | <b>HQ diet</b><br><b>(n = 13)</b> | <b>LQ diet</b><br><b>(n = 11)</b> |                                    |
|---|-----------------------------------|-----------------------------------|------------------------------------|
| <b>Median day to 1<sup>st</sup> egg</b><br><b>(Interquartile range)</b> | 6 (4-6)                           | 8 (4-9)                           | W = 135.0, p = 0.12                |
| <b>Median clutch size (IQR)</b>   | 5 (4.5-7)                         | 2 (2-3)                           | W = 226.0, P = 0.0003**            |
| <b>Mean egg mass per clutch g (SE)</b>                                  | 1.25 (0.04)                       | 1.12 (0.04)                       | F <sub>1,23</sub> = 1.85, p = 0.19 |

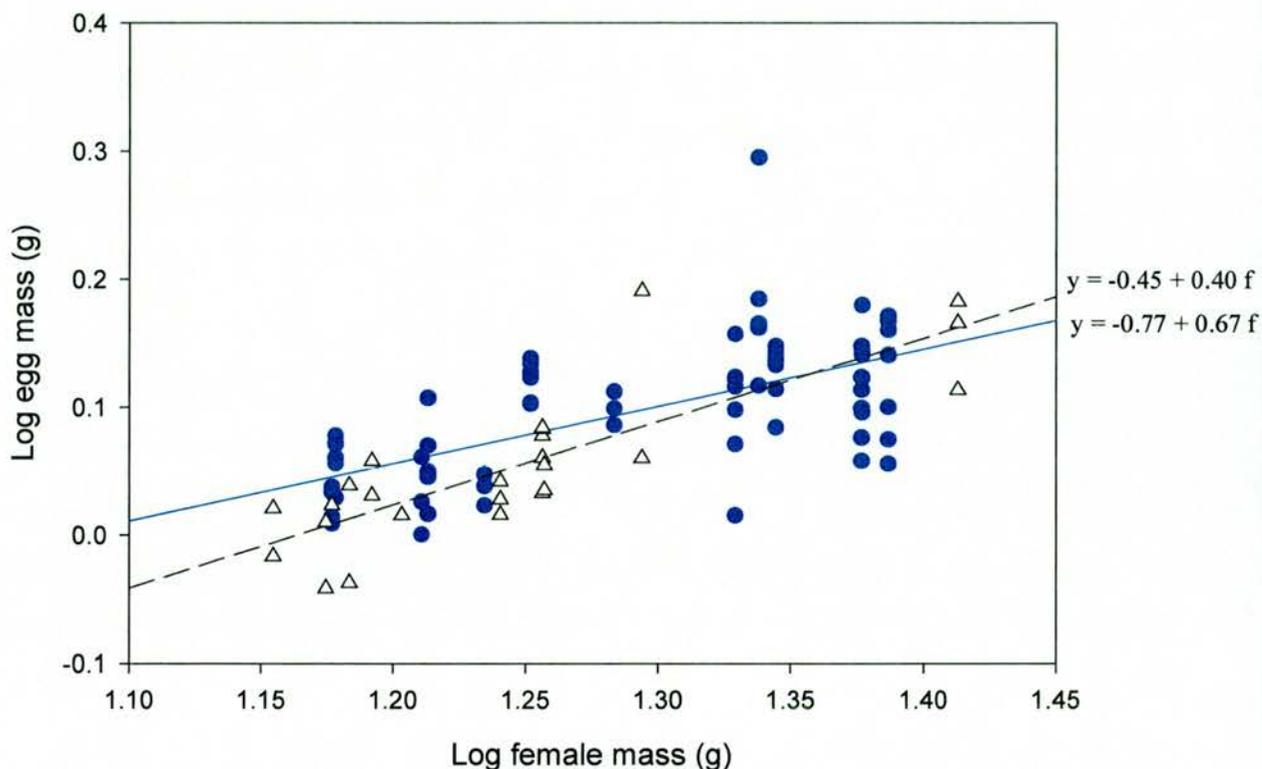


Figure 1. Allometric relationship between log female mass and log egg mass for females that laid on the HQ diet ( $n = 13$ ) and on the LQ diet ( $n = 11$ ). The regression slopes are given in the form:  $y = a + b f$ , where  $y = \log$  egg mass,  $f = \log$  female mass. They are calculated from the GLM, which controls for the change in egg mass with position in the laying sequence. Females on the HQ diet are shown by blue circles and the blue, solid regression line. Females on the LQ diet are shown by white triangles and the black, dashed regression line.

was a significant interaction between position in the laying sequence and diet on egg mass ( $F_{1,52} = 5.72$ ,  $p = 0.02$ , Fig. 2). Egg mass increased with laying sequence on the HQ diet ( $F_{1,44} = 11.07$ ,  $p = 0.002$ ), but there was no effect of laying sequence on egg mass on the LQ diet ( $F_{1,11} = 0.19$ ,  $p = 0.67$ ). On the HQ diet there was no difference in the mass of male and female eggs ( $F_{1,30} = 1.14$ ,  $p = 0.29$ ). On the LQ diet no eggs were sexed (see below).

### **Hatching success**

None of the birds in the low quality group hatched out any chicks. Eggs were either infertile or 'disappeared', presumably eaten by the females. In contrast, 77% (10/13 clutches) of the high quality group hatched young.

### **Sex ratios**

In the HQ diet group, the proportion of male and female chicks did not differ from parity (Table 2). The mean brood sex ratio  $\pm$  SE at laying was:  $0.60 \pm 0.10$ ,  $n = 10$ , at hatching:  $0.66 \pm 0.11$ ,  $n = 10$ , and at fledging:  $0.63 \pm 0.14$ ,  $n = 8$ . There was no effect of laying sequence on the sex of eggs ( $F_{1,37} = 0.87$ ,  $p = 0.36$ ).

### **Mortality**

Embryo mortality was not affected by egg mass ( $F_{1,34} = 0.05$ ,  $p = 0.85$ ) position in the laying sequence ( $F_{1,36} = 0.02$ ,  $p = 0.89$ ), or embryo sex ( $F_{1,37} = 1.29$ ,  $p = 0.26$ ). Post-hatching mortality was not affected by brood size ( $F_{1,28} = 0.30$ ,  $p = 0.59$ ), chick sex ( $F_{1,31} = 0.66$ ,  $p = 0.42$ ), egg mass ( $F_{1,32} = 1.77$ ,  $p = 0.19$ ) or position in the laying sequence ( $F_{1,33} = 3.32$ ,  $p = 0.08$ ).

### **Offspring development**

Hatching mass was highly correlated with egg mass (Table 3). The age at fledging, fledging mass and adult mass were not affected by position in the laying sequence or chick sex (Table 3). However, females had significantly longer tarsi than males at fledging and as adults (Fig. 3).

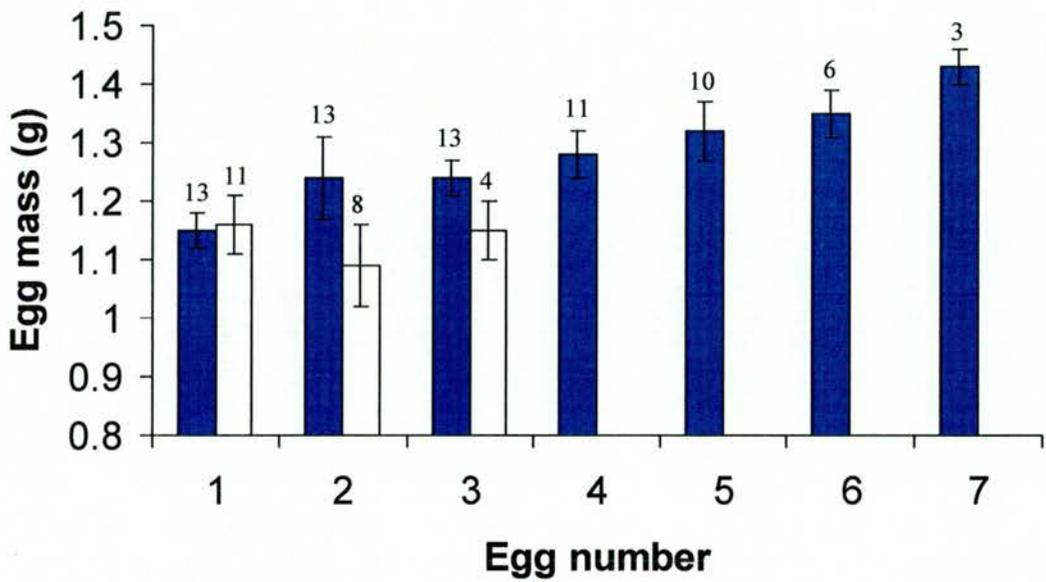


Figure 2. Change in egg mass with position in the laying sequence, showing means and SE. Blue bars represent eggs laid by females on the HQ diet (n = 13). White bars represent eggs laid by females on the LQ diet (n = 11). Numbers of eggs are given above bars.

Table 2. The number of chicks of each sex at laying, hatching and fledging for females on the HQ diet. Sex ratio = males / (males + females).  $G_{adj 1}$  values are goodness of fit against the Mendelian expectation of equal numbers of both sexes. M = males, F = females.

| <b>Laying</b> |       | <b>Hatching</b> |             |            | <b>Fledging</b> |           |             |            |       |           |             |
|---------------|-------|-----------------|-------------|------------|-----------------|-----------|-------------|------------|-------|-----------|-------------|
| No. broods    | M/F   | Sex ratio       | $G_{adj 1}$ | No. broods | M/F             | Sex ratio | $G_{adj 1}$ | No. broods | M/F   | Sex ratio | $G_{adj 1}$ |
| 10            | 27/21 | 0.56            | 0.74        | 10         | 24/20           | 0.55      | 0.36        | 8          | 18/11 | 0.62      | 1.68        |

Table 3. Factors affecting the development of offspring reared on the HQ diet (n = 10 pairs). Non-significant terms were sequentially removed from the model, starting with the least significant interaction terms.

| Trait                | Laying sequence |      | Sex  |          | Egg mass |            |
|----------------------|-----------------|------|------|----------|----------|------------|
|                      | df              | F    | df   | F        | df       | F          |
| Hatching mass (g)    | 1,26            | 1.47 | 1,37 | 0.16     | 1,26     | 33.41      |
|                      |                 | 0.24 |      | 0.69     |          | < 0.0001** |
| Days to fledging     | 1,15            | 0.04 | 1,22 | 0.92     | 1,10     | 0.02       |
|                      |                 | 0.84 |      | 0.34     |          | 0.88       |
| Fledging mass (g)    | 1,7             | 1.43 | 1,15 | 0.00     | 1,21     | 0.00       |
|                      |                 | 0.27 |      | 0.98     |          | 0.95       |
| Fledging tarsus (mm) | 1,13            | 1.46 | 1,18 | 23.10    | 1,14     | 0.23       |
|                      |                 | 0.25 |      | 0.0001** |          | 0.64       |
| Adult mass (g)       | 1,14            | 0.00 | 1,21 | 0.96     | 1,7      | 0.74       |
|                      |                 | 0.96 |      | 0.34     |          | 0.42       |
| Adult tarsus (mm)    | 1,15            | 0.09 | 1,23 | 8.17     | 1,18     | 2.73       |
|                      |                 | 0.77 |      | 0.009**  |          | 0.12       |

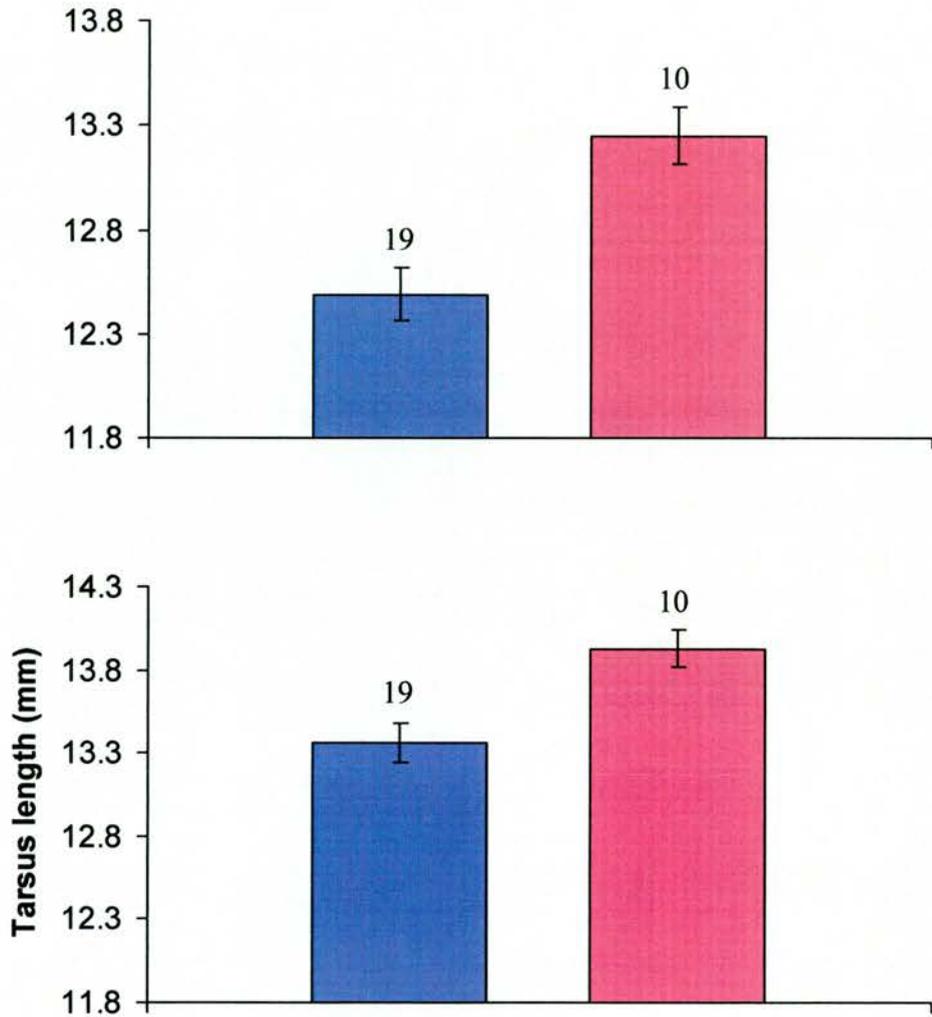


Figure 3. Tarsus length (mean  $\pm$  SE) at (a) fledging and (b) at adulthood of offspring reared on the HQ diet. Blue bars represent males and pink bars represent females. Number of birds are given above bars.

## **Discussion**

The laying data for the two diets were qualitatively similar to the main experiment. Egg mass increased with position in the laying sequence on the HQ diet but not on the LQ diet. In contrast to the main experiment, there was no tendency towards a female-biased sex ratio, but this might be because it was not as high quality a diet. Previous zebra finch studies have also not found a female-biased sex ratio on a high quality or unrestricted diet (Bradbury & Blakey 1998, Kilner 1998). Comparisons could not be made with the LQ diet due to their poor breeding success. Females abandoned their eggs or ate them, and so aborted their efforts at an early stage, presumably because the eggs laid were of too low quality to be successful, or because the females' condition was too low to breed on such a diet.

On the HQ diet, females had longer tarsi at fledging and as adults (in the main experiment females were heavier as adults), which may reflect a greater investment in the sex with the highest fitness returns on a HQ diet. (Kilner 1998).

## Chapter 5: The effect of diet quality on yolk hormone levels

### Introduction

Diet and maternal condition are key predictors of avian reproductive success. When resources are abundant, females are predicted to make relatively large reproductive investments because per unit costs are relatively low and per unit benefits relatively high (Hochachka 1992, Pilz *et al.* 2003). Such large investments are typically reflected in larger clutches and eggs (Bolton *et al.* 1992, Houston *et al.* 1983, Potti 1993, Smith *et al.* 1993), but they may also take the form of more subtle investment such as eggs with greater concentrations of carotenoids, immunoglobulins and hormones, or investment in the more costly sex (Blount *et al.* 2002a, Saino *et al.* 2002, Pilz *et al.* 2003, Appleby *et al.* 1997, respectively).

In zebra finches, *Taeniopygia guttata*, females are protein-limited during egg production (Houston *et al.* 1995, Williams 1996a) such that, females on a protein-rich diet have been found to lay more and larger eggs (Monaghan *et al.* 1996, Selman & Houston 1996, Williams 1996a, Rutkowska & Cichon 2002, this thesis Chapter 4). Additionally, on a protein-rich, high quality (HQ) diet hatching success was found to be greater than on a low quality (LQ) diet (Selman & Houston 1996, Gorman & Nager 2003, this thesis Chapter 4).

I have also found previously (this thesis Chapter 4) that, diet quality affected the pattern of intra-clutch variation in egg mass. On a HQ diet egg mass increased with position in the laying sequence while on a LQ diet egg mass tended to decrease with position in the laying sequence. In asynchronously hatching species, an increase in egg mass with position in the laying sequence when conditions are favourable might represent a brood survival strategy (Howe 1976, Clark & Wilson 1981, Slagsvold *et al.* 1984), whereby females invest more in later eggs to offset the disadvantage to these chicks of hatching last. In contrast, a decline in egg mass with laying sequence when conditions are harsh might represent a brood reduction strategy (Parsons 1970, O'Connor 1979, Lundberg & Vaisainen 1979, Alikauskas 1986) since hatching at a smaller mass further disadvantages late-hatching chicks.

### Maternally-derived yolk androgens

A second possible mechanism by which females might adjust the within-brood competitive hierarchy is by differentially provisioning egg yolks with androgens. In birds, lipid-soluble steroids pass from the mother into the yolk during vitellogenesis, and it is thought that the female has the potential to control how much each egg is allocated. The amount of testosterone (T) and 5 $\alpha$ -dihydrotestosterone (DHT) an egg receives relative to the rest of the clutch may have important fitness consequences. In canaries, *Serinus canaria*, the social rank of juvenile was positively correlated with the concentration of yolk T in the eggs from which they hatched (Schwabl 1993), and injections of T into the yolk were found to confer a competitive advantage to chicks by enhancing begging behaviour, post-hatching growth and development rates (Schwabl 1996a).

Similar effects of T have been reported in other species. First-laid eggs of black-headed gulls were injected with either an androgen solution or oil control, and artificial broods were then created comprising only first laid eggs. Androgen treatment was found to enhance embryonic development and post-hatching growth, as well as increasing competitive ability (Eising *et al.* 2001).

In red-winged blackbirds, *Agelaius phoeniceus*, yolk T concentration and mass of the complexus (a neck muscle, thought to be important in breaking the shell and in begging) were both found to increase with position in the laying sequence (Lipar & Ketterson 2000). The injection of T directly into egg yolks resulted in an increase in relative complexus mass and, conversely, the injection of a T antagonist resulted in a decrease in relative complexus mass.

However, in other species T had been found to have deleterious effects. In American kestrels, *Falco sparverius*, yolks of eggs laid later in the laying sequence were found to contain more T and androstenedione (A<sub>4</sub>) compared with first-laid eggs (Sockman & Schwabl 2000). When early eggs were injected with T levels similar to later laid eggs, this was found to delay hatching and reduce nestling growth and survival rates. A study of house sparrows, *Passer domesticus*, also found that yolk T levels were negatively correlated with hatching success (Mazuc *et al.* 2003). Steroid hormones are thought to impair enzymatic antioxidant defences and directly induce oxidative stress (von Schantz *et al.* 1999) and so the balance between beneficial and deleterious effects may

depend both on the level of yolk androgens and the characteristics of the egg (Groothuis & Schwabl 2002).

To date, yolk steroids have been studied in only a handful of avian species, revealing many different patterns. The levels of T differ vastly in different species, irrespective of yolk volume. Levels are relatively low in zebra finches (Schwabl 1993, Gil *et al.* 1999), tree swallows, *Tachycineta bicolor* (Whittingham & Schwabl 1993) and European starlings, *Sturnus vulgaris* (Pilz *et al.* 2003) and higher in canaries (Schwabl 1993), house sparrows (Schwabl 1997, Mazuc *et al.* 2003), and red-winged blackbirds (Lipar *et al.* 1999).

Within-clutch androgen deposition has also been found to vary systematically with position in the laying sequence within the clutch between species. In the majority of species studied to date, yolk T has been found to increase with laying sequence; canaries (Schwabl 1993), red-winged blackbirds (Lipar *et al.* 1999), American kestrels (Sockman & Schwabl 2000), lesser black-backed gulls (Royle *et al.* 2001), common terns, *Sterna hirundo* (French *et al.* 2001), black-headed gulls (Groothuis & Schwabl 2002, Eising *et al.* 2001) and European starlings (Pilz *et al.* 2003).

Decreasing levels of yolk T with laying sequence have so far been reported in only two species; cattle egrets, *Bubulcus ibis* (Schwabl *et al.* 1997) and zebra finches (Gil *et al.* 1999). In house wrens, *Troglodytes aedon*, and tree swallows, no relationship between yolk T and laying sequence was found (Ellis *et al.* 2001, Whittingham & Schwabl 2002).

These within-clutch species differences in yolk androgens are possibly adaptive, reflecting mechanisms to promote or reduce brood reduction in asynchronously hatching species. In species where T increases with position in the laying sequence, this has generally been interpreted as a compensatory maternal mechanism to mitigate the disadvantage to last-hatching chicks (Schwabl 1993, 1996a). Cattle egrets, on the other hand, are a facultative siblicidal species and so the provisioning of first laid eggs with extra T was thought to facilitate efficient brood reduction by the oldest chicks (Schwabl *et al.* 1997). Zebra finches are not siblicidal but do live in arid areas where high quality food may be in short supply (Zann 1996) and so a mechanism to promote brood reduction may also have evolved in this species. However, in certain seasons breeding conditions may be

favourable, especially following heavy rains, and so brood reduction may not always be adaptive. Therefore, some degree of flexibility is expected.

Yolk T concentrations are positively correlated with circulating testosterone levels in the female during yolk formation (Schwabl 1996b) and differences in yolk androgen levels within species have been found to relate to the social conditions of the mother. Yolk androgen levels were found to correlate with the density of the breeding colony in house sparrows (Schwabl 1997) and with the number of aggressive interactions in colonies of black-headed gulls (Groothuis & Schwabl 2002) and in tree swallows (Whittingham & Schwabl 2002). Therefore, it has been proposed that differential allocation of maternally-derived yolk androgens may function to modify offspring development to the environmental conditions experienced by the mother, and to which later the offspring will be subjected (Schwabl 1996b).

Androgen levels may also be affected by female quality. In European starlings androgen levels were positively correlated with female clutch size and laying date as well as female age (Pilz *et al.* 2003). However, Groothuis & Schwabl (2002) found that, in black-backed gulls, eggs of lighter clutches contained greater concentrations of T. The authors suggested that these eggs were probably laid by younger females, and that they may have compensated for the lower quality of their eggs by increasing their androgen content. A compensation effect was also found with respect to yolk carotenoid concentrations in barn swallows, when females were mated to males with experimentally-shortened tails (Saino *et al.* 2002).

In zebra finches flexibility in yolk androgen deposition was investigated in relation to mate attractiveness. Females were found to deposit more T and DHT into their eggs when paired with an attractive male (Gil *et al.* 1999). The authors proposed that androgens may be costly to either the laying female or to her chicks (see Sockman & Schwabl 2000, Royle *et al.* 2001, Mazuc *et al.* 2003). Therefore, only high quality offspring may be able to withstand or are worth this greater investment.

### **Differential sex allocation**

Yolk androgens may also be deposited differentially in male and female eggs when, for example, the impact of a given unit of parental investment enhances the reproductive value

of one offspring sex more than the other (Trivers & Willard 1973, Leimar 1996). In Leghorn chickens, *Gallus gallus domesticus*, the relationship between embryo sex and androgen levels has been found to depend on the social rank of the female. Dominant females were found to allocate more yolk T to male eggs, whereas subordinate females allocated more T to female eggs (Müller *et al.* 2002). Sex allocation theory predicts such a pattern of resource allocation because in this polygynous species males have higher condition-dependent variance in reproductive success than do females.

Zebra finches are socially monogamous and size monomorphic, and therefore, differential investment in the sexes might not seem likely. However, when food conditions are poor, sex ratio skews towards males have been found at laying (Rutkowska & Cichon 2002, this thesis Chapter 4) and at hatching (Bradbury & Blakey 1998, Kilner 1998). This is thought to be adaptive because female fecundity (clutch mass) is strongly related to female weight at fledging (Haywood & Perrins 1992) and females also have lower rates of growth and survival on a poor quality diet (Zann & Runciman 1994, de Kogel 1997, Bradbury & Blakey 1998, Kilner 1998, Birkhead *et al.* 1999). The reason for this is not known, but it cannot be solely due to sibling competition or directed parental provisioning because females have been found to gain mass more slowly than males when hand-reared on a restricted diet (Martins 2003). It is possible that such differences in male and female growth rates are mediated through sex differences in yolk hormone levels or by sex differences in sensitivity to yolk hormone levels (Henry & Burke 1999).

### **Aims**

This chapter aimed firstly to investigate differences in levels of yolk androgen deposition in relation to diet quality. If androgens are costly to the female or to her offspring, females on a HQ diet were predicted to invest more than those on a LQ diet. Alternatively, females on a LQ diet may attempt to compensate for the poorer quality of their eggs (Groothuis & Schwabl 2002) and therefore the converse may be predicted, i.e. androgen levels may be higher on the LQ diet.

Secondly, it aimed to investigate differences in within-brood patterns of androgen deposition. Since diet quality affects the number and quality of young that females can rear, females on a HQ diet were predicted to deposit relatively more T and DHT in later

eggs (brood survival strategy), whereas on a LQ diet, females were predicted to deposit relatively less T and DHT in later eggs (brood reduction strategy). This would follow the same pattern as intra-clutch patterns in egg mass found in this species (this thesis Chapter 4).

Thirdly, this chapter aimed to investigate possible sex differences in yolk androgen levels. If female reproductive success is more dependent on nestling resources than that of males, then on a HQ diet female eggs were predicted to contain greater concentrations of T and DHT and, on a LQ diet male eggs were predicted to contain greater concentrations of T and DHT.

## Methods

### Experimental design

Forty female zebra finches were randomly assigned to one of two diet groups for six weeks prior to breeding. Females on the HQ regime were given foreign finch mix *ad libitum* (approx. 14% protein), supplemented daily with Haith's egg biscuit food (13.2% protein), mixed with hard-boiled hen's egg (13.5% protein), PBX protein crumb (26% protein) and fresh spinach. Females on the LQ regime were given foreign finch mix *ad libitum* and fresh spinach three times a week. Both groups had access to cuttlefish bone and fresh water *ad libitum*.

Females were weighed after six weeks and paired randomly with males with whom they had not previously bred. The pairs were placed in individual breeding cages, equipped with open nest boxes and nesting material. For the six weeks prior to pairing, all males had been caged in single sex groups in a separate room, on the standard laboratory diet of foreign finch mix, supplemented with fresh spinach and egg mix once a week. Once paired, males were kept on the same HQ or LQ diet as their mate and these diets were maintained during egg laying and incubation.

Nests were checked every morning for eggs. Newly laid eggs were removed, weighed to the nearest 0.01g, and numbered with a non-toxic pen. Eggs were replaced with dummy eggs to maintain the clutch size. Eggs were artificially incubated at 37.5°C for 72 hours to enable an embryo large enough for sexing to develop. At this stage eggs were removed from the incubator and stored at -20°C for later dissection.

After one month, pairs were separated, re-weighed and housed in single sex groups. After a two-week rest period (during which females received the standard laboratory diet) the experimental diets were reversed. Females that were formerly given the HQ diet received the LQ diet, and vice-versa. Males were once again given the standard laboratory diet. After six weeks females were paired up with the same male as in round one and the experiment was repeated.

### **Egg dissection**

The embryo was removed from fertile eggs and stored at  $-20^{\circ}\text{C}$  for DNA extraction and sexing (see Chapter 2). The yolk was separated from the white and stored at  $-20^{\circ}\text{C}$  for steroid extractions (see Chapter 2). Eggs in which there was no sign of embryonic development were presumed to be infertile.

### **Radioimmunoassays**

See Chapter 2 for methods. The intra-assay coefficient of variation was  $4.3 \pm 0.3\%$  (mean  $\pm$  SE) for T and  $6.3 \pm 0.6\%$  for DHT. The inter-assay coefficient of variation was  $17 \pm 2.2\%$  for both. The extraction recovery of total (T + DHT) androgens was  $75.6 \pm 9.0\%$ ; extraction recovery of DHT only was  $59 \pm 0.9\%$ . The cross-reactivity of the antisera was 46% (see also Nash *et al.* 2000), so the T content was estimated as total-(0.46 x DHT).

### **Statistical analyses**

Total yolk androgens were calculated by multiplying initial yolk mass by androgen concentration. Because the development of an embryo disrupts the distinction between egg white and yolk, I estimated the initial yolk mass from the whole egg mass, using the regression equations obtained from data on the composition of fresh eggs laid on HQ and LQ diets (Chapter 6). This has the advantage that yolk mass would relate to initial yolk mass (prior to embryonic development) and thus make no assumptions concerning the rate of development of the embryo, which would in turn also affect the mass of the remaining yolk. The regression equations were: for HQ diet eggs,  $y = 0.028 + (0.145 e)$ ; for LQ eggs,  $y = 0.045 + (0.129 e)$ , where  $y$  = yolk mass,  $e$  = egg mass.

Analyses were performed using the repeated measures general linear model (GLM) procedure described in Chapter 2. Paired data were analysed for females that laid eggs on both diets. Non-normal data were log-transformed ( $\log(\text{androgen value} + 1)$ ). Since values for androgen concentration and total androgen levels gave very similar results, statistical test results are given only for androgen concentration.

## Results

### Clutch size and egg mass

Thirty out of 40 females laid a clutch on both the HQ and LQ diets (17 females laid a clutch on the HQ diet in round one and on the LQ diet in round two, and 13 females laid a clutch on the LQ diet in round one and on the HQ diet in round two). Seven females did not lay a clutch on either diet, two laid only on the HQ diet and one laid only on the LQ diet.

Paired analyses were carried out on the 30 females that laid a clutch on both diets. Females that were on HQ diet first began laying significantly sooner than when on the LQ diet, but there was no significant difference in the number of days between pairing and laying of the first egg for females that laid on the LQ diet first (Fig. 1). Regardless of order of treatment, females laid significantly larger clutches and heavier eggs when on the HQ diet (Figs. 2 & 3).

### Intra-clutch variation in egg mass

Egg mass was analysed in relation to diet, breeding round, female mass, position in the laying sequence and embryo sex (Table 1). There was a significant interaction between position in the laying sequence and diet with respect to egg mass both for females that laid on the HQ diet first ( $F_{1,38} = 14.28$ ,  $p = 0.0005$ , Fig. 4a) and for females that laid on the LQ diet first ( $F_{1,107} = 4.00$ ,  $p = 0.048$ , Fig. 4b). Egg mass increased with egg number significantly for females on the HQ diet in round one ( $F_{1,74} = 4.68$ ,  $p = 0.03$ , Fig. 4a), and for females on the HQ diet in round two ( $F_{1,65} = 5.47$ ,  $p = 0.02$ , Fig. 4b). In contrast, egg mass tended to decrease with laying sequence for females that laid on the LQ diet in round two ( $F_{1,46} = 3.94$ ,  $p = 0.053$ , Fig. 4a) and did not change for females that laid on the LQ diet in round one ( $F_{1,37} = 1.20$ ,  $p = 0.28$ , Fig. 4b).

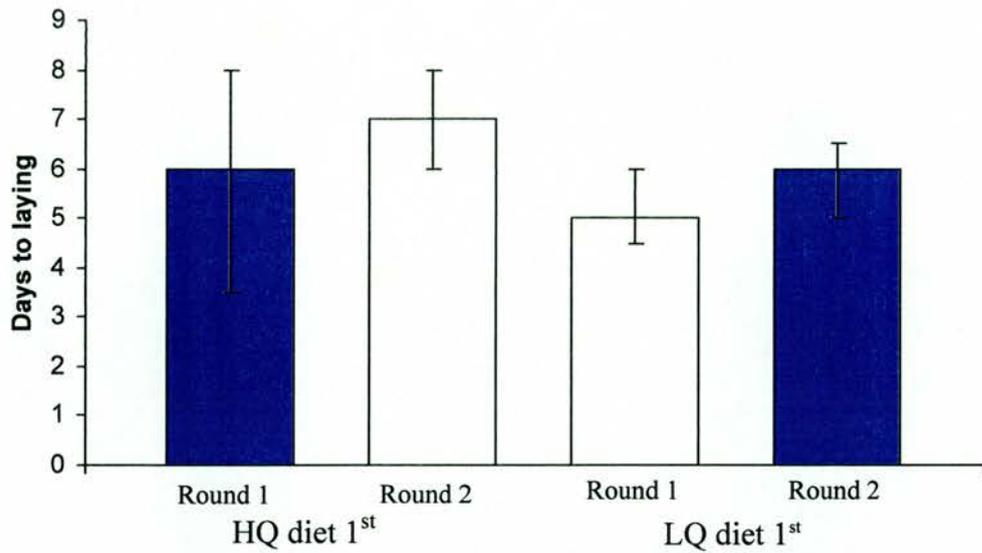


Figure 1. Median number of days (error bars are interquartile range) between pairing and laying of the first egg. Females that laid on the HQ diet first (n = 17): Wilcoxon signed ranks test,  $W = 18.0$ ,  $p = 0.03$ . Females that laid on the LQ diet first (n = 13):  $W = 15$ ,  $p = 0.41$ .

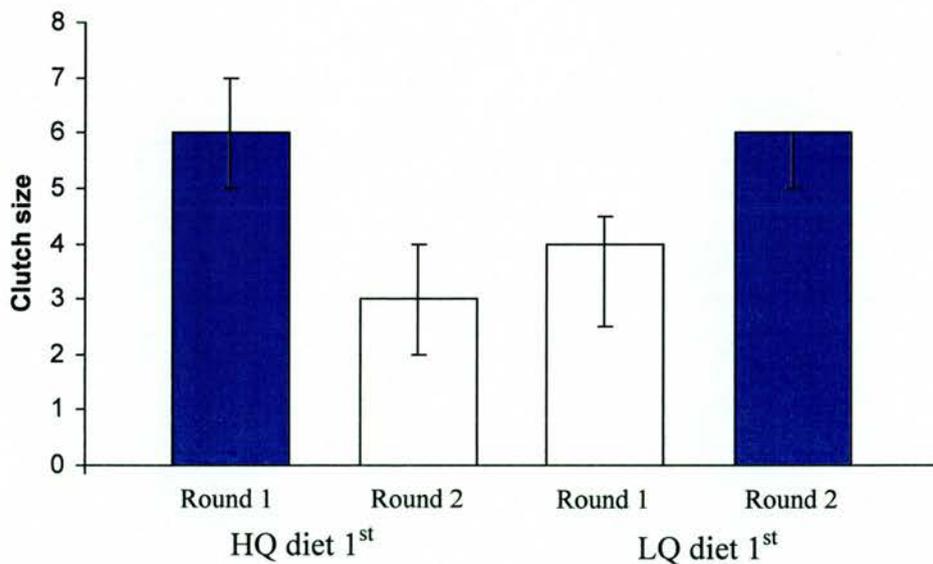


Figure 2. Median clutch sizes (error bars are interquartile range). Clutches were significantly larger on the HQ diet than on the LQ diet: Females that laid on the HQ diet first (n = 17):  $W = 120.0$ ,  $p = 0.001$ , Females that laid on the LQ diet first (n = 13):  $W = 0.00$ ,  $p = 0.006$ .

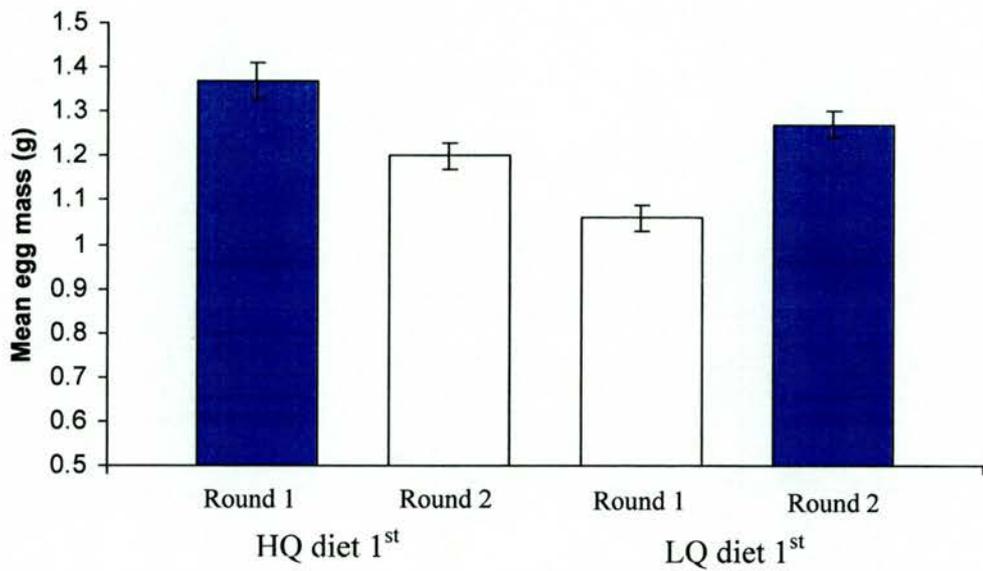


Figure 3. Mean egg mass per clutch ( $\pm$  S.E). Eggs were significantly heavier on the HQ diet than on the LQ diet: Females that laid on the HQ diet first ( $n = 17$ ): Paired t-test,  $t = 6.06$ ,  $p < 0.001$ . Females that laid on the LQ diet first ( $n = 13$ ):  $t = 4.44$ ,  $p = 0.001$ . Female mass did not significantly affect egg mass and so was not included as a covariate.

Table 1. Factors influencing the mass of eggs (paired GLM analysis). Non significant interactions were removed from the model and hence not shown. Paired data were analysed for 30 females (273 eggs) that laid on both the HQ and LQ diets.

| <b>Variable</b>                     | <b>df</b> | <b>F</b> | <b>p</b> |
|-------------------------------------|-----------|----------|----------|
| <b>Diet</b>                         | 1,101     | 0.04     | 0.85     |
| <b>Female mass</b>                  | 1,57      | 2.71     | 0.11     |
| <b>Breeding round</b>               | 1,52      | 4.06     | 0.049    |
| <b>Laying sequence</b>              | 1,123     | 0.47     | 0.50     |
| <b>Female mass * breeding round</b> | 1,52      | 4.46     | 0.04     |
| <b>Diet * breeding round</b>        | 1,57      | 8.34     | 0.006    |
| <b>Diet * laying sequence</b>       | 1,53      | 16.50    | 0.0002   |

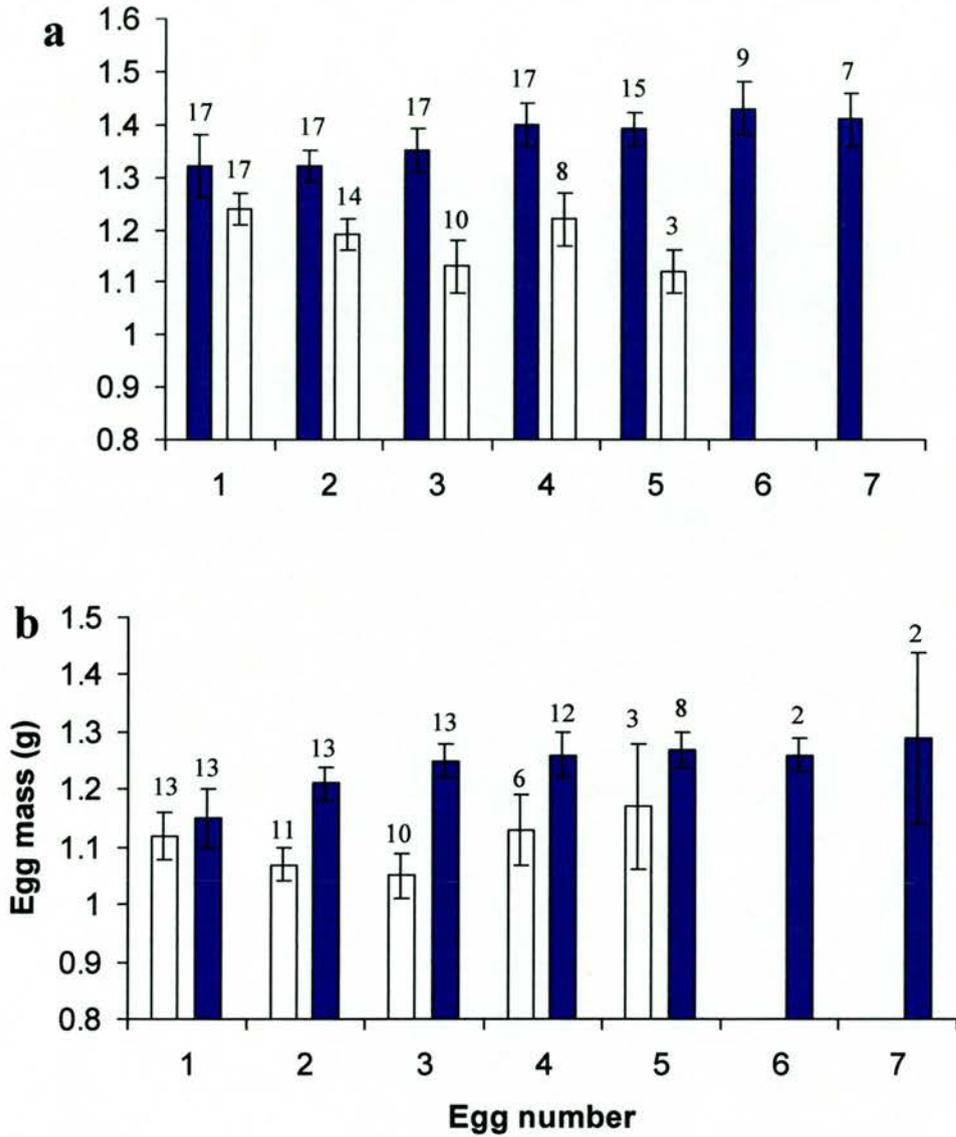


Figure 4. Mean egg mass ( $\pm$  SE) in relation to position in the laying sequence. Data are paired for females that laid (a) on the HQ diet in round one ( $n = 17$ ), and (b) on the LQ diet in round one ( $n = 13$ ). Blue bars represent HQ diet eggs and white bars represent LQ diet eggs. Numbers of eggs are given above bars.

There was also a significant interaction between breeding round and diet with respect to egg mass (Table 1, Figs. 3 & 4). Females that were on the HQ diet in the first round laid heavier eggs than females that were on the HQ diet in round two. Unexpectedly, they continued to lay heavier eggs on the LQ diet (their second experimental diet) compared with the females that laid on the LQ diet in round one.

In addition, there was a significant interaction between breeding round and female mass (Fig. 5). Female mass had a significant positive effect on egg mass only in the first round of breeding ( $F_{1,25} = 7.19$ ,  $p = 0.01$ ), although this was significant for the HQ diet females ( $F_{1,14} = 5.00$ ,  $p = 0.04$ ), but not for the LQ diet females ( $F_{1,12} = 1.12$ ,  $p = 0.31$ ). Female mass did not have a significant effect on egg mass in the second round of breeding for either the HQ diet females ( $F_{1,11} = 0.02$ ,  $p = 0.88$ ) or the LQ diet females ( $F_{1,15} = 0.75$ ,  $p = 0.40$ ). There was no difference in the mass of male and female eggs ( $F_{1,120} = 0.80$ ,  $p = 0.37$ ) and the interaction between diet and embryo sex was not significant ( $F_{1,121} = 0.31$ ,  $p = 0.58$ ).

### **Egg fertility**

In round one the proportion of fertile eggs on the HQ diet was significantly higher than on the LQ diet: 82% (94/115) of the HQ diet eggs were fertile, and 64% (30/47) of the LQ diet eggs ( $G = 5.56$ ,  $df = 1$ ,  $p < 0.02$ ). Out of the fertile eggs, it was possible to sex 76% ( $n = 71$ ) of the HQ diet eggs and 83% ( $n = 25$ ) of the LQ diet eggs.

In round two, there was no significant difference in the proportion of fertile eggs laid on the two diets: 87% (61/70) of the HQ diet eggs were fertile, and 85% (44/52) of the LQ diet eggs ( $G = 0.15$ ,  $df = 1$ ,  $p > 0.5$ ). Of these, it was possible to sex 92% ( $n = 56$ ) of the HQ diet eggs and 85% ( $n = 37$ ) of the LQ diet eggs.

### **Sex ratios**

The number of male and female eggs produced on each diet in breeding rounds one and two is summarised in Table 2. The sex ratio did not differ significantly from 50:50 in either diet. Paired analyses were carried out on the mean clutch sex ratio was for 21 females that laid fertile clutches on both diets (14 females that were on the HQ diet and

Figure 5 (overleaf). Allometric relationship between log female mass and log egg mass for females that laid (a) on the HQ diet in round one and the LQ diet in round two ( $n = 17$ ) and (b) on the LQ diet in round one and the HQ diet in round two ( $n = 13$ ). The regression slopes are given in the form:  $y = a + b f$ , where  $y = \log$  egg mass,  $f = \log$  female mass. They are calculated from the GLM, which controls for the change in egg mass with laying sequence. Females on the HQ diet are shown by blue circles and blue, solid regression lines. Females on the LQ diet are shown by white triangles and black, dashed regression lines.

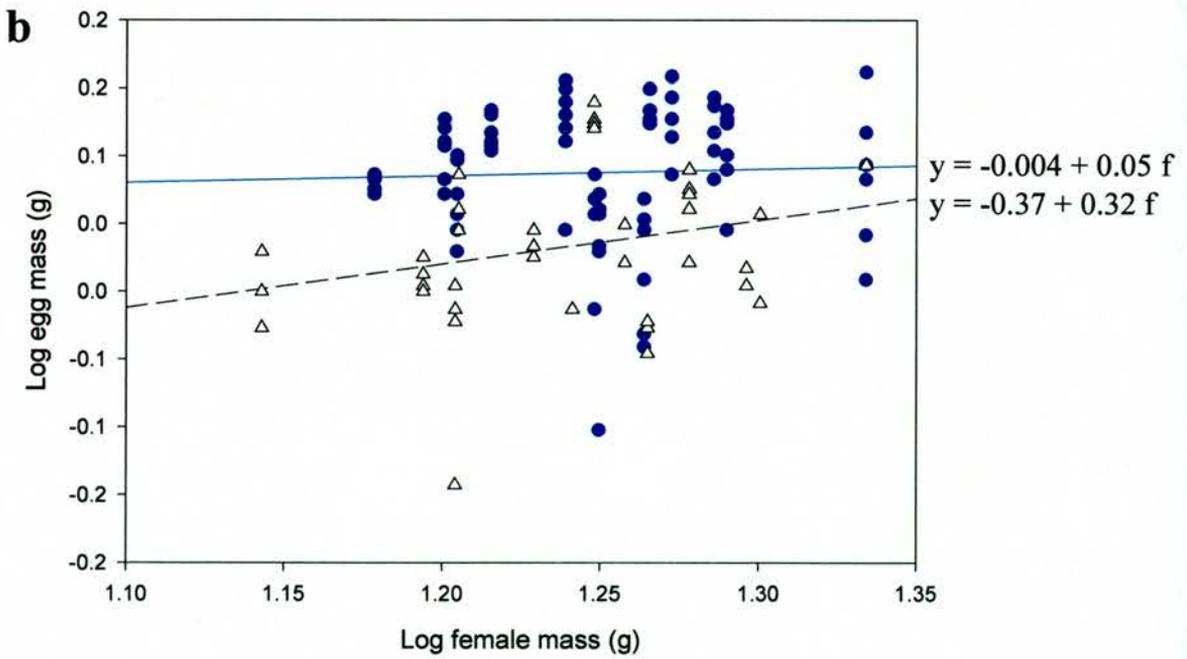
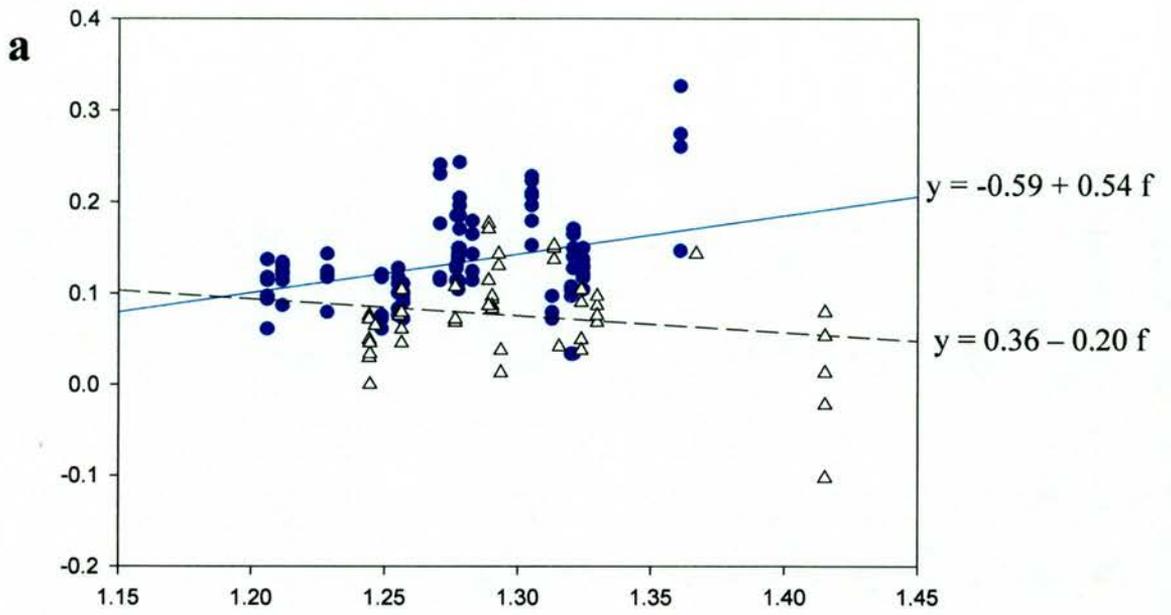


Table 2. The number of male and female eggs produced by females on the HQ and LQ diets in round one and round two. Sex ratio = males / (males + females). All females in round two had also laid clutches in round one.  $G_{adj 1}$  values are goodness of fit against the Mendelian expectation of equal numbers of both sexes. M = males, F = females.

|                | Round one  |       |           | Round two   |            |       |           |             |
|----------------|------------|-------|-----------|-------------|------------|-------|-----------|-------------|
|                | No. broods | M/F   | Sex ratio | $G_{adj 1}$ | No. broods | M/F   | Sex ratio | $G_{adj 1}$ |
| <b>HQ diet</b> | 16         | 38/33 | 0.54      | 0.35        | 12         | 26/30 | 0.46      | 0.28        |
| <b>LQ diet</b> | 9          | 14/11 | 0.56      | 0.35        | 14         | 18/19 | 0.49      | 0.03        |

seven that were on the LQ diet in round one). Two other females laid fertile clutches on both diets but over half of their eggs could not be sexed with certainty and so they were excluded from the analysis. There was no difference in the mean clutch sex ratio between the two diets (mean clutch sex ratio  $\pm$  SE, HQ diet:  $0.48 \pm 0.07$ , LQ diet:  $0.51 \pm 0.08$ ,  $F_{1,20} = 0.10$ ,  $p = 0.76$ ). There was no effect of order of treatment ( $F_{1,19} = 0.01$ ,  $p = 0.92$ ). Finally, there was no effect of laying sequence on embryo sex ( $F_{1,108} = 2.04$ ,  $p = 0.16$ ) and the interaction between laying sequence and diet was not significant ( $F_{1,107} = 0.06$ ,  $p = 0.81$ ).

### **Androgen analyses**

#### *Correlation of egg size and androgen levels*

Yolk mass was not significantly correlated with either T concentration ( $F_{1,137} = 2.62$ ,  $p = 0.11$ ) or DHT concentration ( $F_{1,84} = 0.11$ ,  $p = 0.74$ ).

#### *Androgen levels: fertile versus infertile eggs*

Factors influencing T and DHT concentration are summarized in Table 3. There was a significant three-way interaction between diet, fertility and breeding round for both T concentration (Fig. 6a) and DHT concentration (Fig. 6b). For females that laid on the HQ diet in round one (and therefore the LQ diet in round two), infertile eggs contained more T ( $F_{1,122} = 16.51$ ,  $p < 0.0001$ , Fig. 6a) and more DHT ( $F_{1,131} = 5.03$ ,  $p = 0.03$ , Fig. 6b) than did fertile eggs. There was no effect of diet on T ( $F_{1,18} = 0.28$ ,  $p = 0.60$ ) or DHT ( $F_{1,86} = 0.31$ ,  $p = 0.58$ ) concentration, and no interaction between diet and fertility for T ( $F_{1,69} = 0.57$ ,  $p = 0.45$ ) or DHT ( $F_{1,97} = 2.16$ ,  $p = 0.14$ ).

However, for females that laid on the LQ diet in round one (and therefore the HQ diet in round one), there was no difference between infertile and fertile eggs for either T ( $F_{1,104} = 0.43$ ,  $p = 0.51$ , Fig. 6a) or DHT concentration ( $F_{1,88} = 1.39$ ,  $p = 0.24$ , Fig. 6b). Again, there was no effect of diet on T ( $F_{1,24} = 1.61$ ,  $p = 0.22$ ) or DHT ( $F_{1,18} = 0.28$ ,  $p = 0.60$ ) and no interaction between diet and fertility for T ( $F_{1,102} = 0.43$ ,  $p = 0.51$ ) or DHT ( $F_{1,69} = 0.57$ ,  $p = 0.45$ ).

Table 3. Factors influencing yolk T and DHT concentration of all eggs (GLM analysis). The data included 260 eggs from 30 females that laid a clutch on both diets. Non-significant interactions were removed from the model.

| <b>Variable</b>                          | <b>df</b> | <b>F</b> | <b>p</b> |
|--|-----------|----------|----------|
| <i>T concentration</i>                   |           |          |          |
| <b>Egg number</b>                        | 1,226     | 3.93     | 0.049    |
| <b>Diet</b>                              | 1,78      | 2.08     | 0.15     |
| <b>Breeding round</b>                    | 1,75      | 0.50     | 0.48     |
| <b>Fertility</b>                         | 1,226     | 3.65     | 0.057    |
| <b>Diet * breeding round</b>             | 1,94      | 8.86     | 0.004    |
| <b>Fertility * breeding round</b>        | 1,217     | 0.65     | 0.42     |
| <b>Diet * fertility</b>                  | 1,212     | 0.00     | 0.99     |
| <b>Diet * breeding round * fertility</b> | 1,226     | 7.53     | 0.003    |
| <i>DHT concentration</i>                 |           |          |          |
| <b>Egg number</b>                        | 1,174     | 14.96    | 0.0002   |
| <b>Diet</b>                              | 1,58      | 0.35     | 0.55     |
| <b>Breeding round</b>                    | 1,56      | 0.45     | 0.51     |
| <b>Fertility</b>                         | 1,171     | 0.74     | 0.39     |
| <b>Diet * breeding round</b>             | 1,74      | 4.00     | 0.049    |
| <b>Fertility * breeding round</b>        | 1,154     | 0.21     | 0.65     |
| <b>Diet * fertility</b>                  | 1,151     | 2.97     | 0.09     |
| <b>Diet * breeding round * fertility</b> | 1,167     | 5.86     | 0.02     |

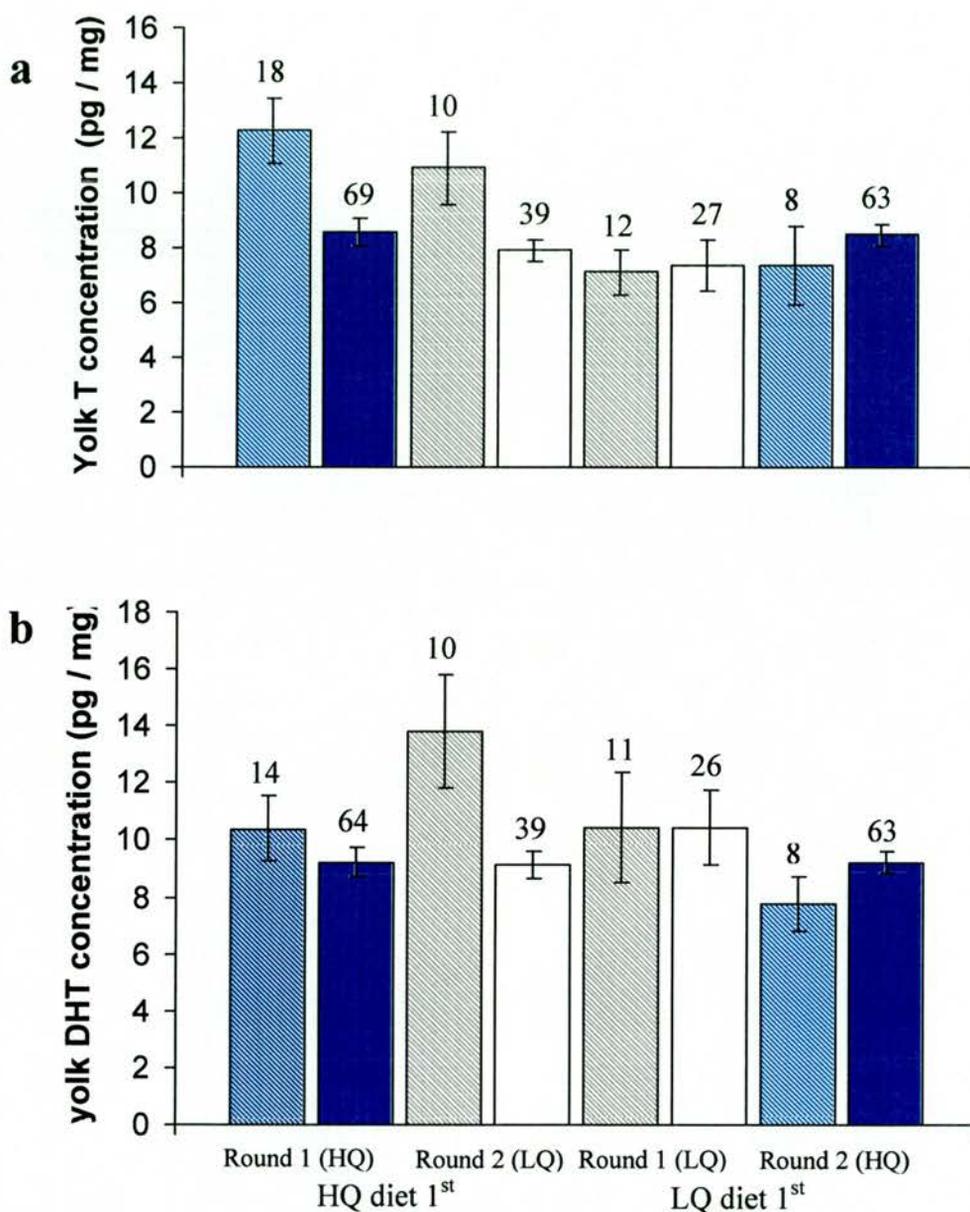


Figure 6. Concentrations (means  $\pm$  SE) of (a) T and (b) DHT for infertile and fertile eggs paired for females that laid on the HQ diet in round one and the LQ diet in round two ( $n = 17$ ), and for females that laid on the LQ diet in round one and the HQ diet in round two ( $n = 13$ ). Number of eggs that were assayed are given above bars. Filled bars represent fertile eggs and hatched bars represent infertile eggs.

*Analyses of fertile eggs: effects of sex and laying sequence*

A paired analysis was conducted for females that laid at least one fertile egg in both clutches ( $n = 23$  females, Table 4). There was a significant interaction between diet, sex and breeding round both for T ( $F_{1,118} = 6.11$ ,  $p = 0.01$ ) and DHT ( $F_{1,136} = 12.63$ ,  $p = 0.0005$ ). For T, but not DHT, there was also an interaction between egg number, sex and breeding round ( $F_{1,125} = 18.32$ ,  $p < 0.0001$ ).

For females that laid on the HQ diet in round one and, therefore the LQ diet in round two ( $n = 14$ ), there was a significant interaction between diet, sex and position in the laying sequence for T concentration ( $F_{1,77} = 10.15$ ,  $p = 0.002$ , Fig. 7). In round one, i.e. when these females were on the HQ diet (Fig. 7a), T in male eggs decreased with position in the laying sequence ( $F_{1,21} = 7.41$ ,  $p = 0.01$ ), whereas T in female eggs increased with position in the laying sequence ( $F_{1,25} = 10.68$ ,  $p = 0.003$ ). In round two, i.e. on the LQ diet (Fig. 7b), T tended to be higher in female eggs than male eggs ( $F_{1,32} = 4.01$ ,  $p = 0.054$ ) and decreased with position in the laying sequence in both sexes ( $F_{1,21} = 4.56$ ,  $p = 0.04$ ). DHT tended to be greater for females than males on both diets ( $F_{1,85} = 3.81$ ,  $p = 0.054$ ) and decreased with position in the laying sequence on both diets ( $F_{1,86} = 6.11$ ,  $p = 0.02$ ). There was no interaction between diet and laying sequence ( $F_{1,84} = 1.02$ ,  $p = 0.32$ ).

For females that laid on the LQ diet in round one and, therefore, the HQ diet in round two ( $n = 9$ ), there was a significant interaction between diet and embryo sex for both T ( $F_{1,71} = 4.28$ ,  $p = 0.04$ , Fig. 8a) and DHT ( $F_{1,72} = 7.75$ ,  $p = 0.007$ , Fig. 8b). When these females were on the HQ diet, i.e. round two, there was no difference between male and female eggs for either T ( $F_{1,54} = 0.00$ ,  $p = 0.97$ ) or DHT ( $F_{1,46} = 0.15$ ,  $p = 0.70$ ) but on the LQ diet, i.e. round one, male eggs contained higher androgen concentrations than female eggs (T:  $F_{1,18} = 4.43$ ,  $p = 0.049$ , DHT:  $F_{1,18} = 5.12$ ,  $p = 0.04$ ). On both diets, androgens decreased with position in the laying sequence for T ( $F_{1,71} = 9.24$ ,  $p = 0.003$ ) and DHT concentration ( $F_{1,68} = 30.91$ ,  $p < 0.0001$ ). Again, there was no interaction between diet and laying sequence for either T ( $F_{1,70} = 2.48$ ,  $p = 0.12$ ) or DHT ( $F_{1,64} = 1.54$ ,  $p = 0.22$ ).

**Female body mass**

Paired analyses were carried out for females that laid a clutch on both diets ( $n = 30$ ). Experimental diet had no effect on female mass during the time on that diet prior to pairing

Table 4. Factors influencing yolk T and DHT concentration of fertile eggs (GLM analysis). The data included 186 eggs from 23 females that laid at least one fertile egg on both diets. Non-significant interactions were removed from the model and hence are not shown.

| Variable                          | df    | F     | p        |
|-----------------------------------|-------|-------|----------|
| <i>T concentration</i>            |       |       |          |
| Egg number                        | 1,129 | 3.38  | 0.07     |
| Diet                              | 1,91  | 12.04 | 0.0008   |
| Breeding round                    | 1,130 | 7.67  | 0.006    |
| Sex                               | 1,115 | 2.15  | 0.15     |
| Diet * breeding round             | 1,46  | 0.24  | 0.63     |
| Sex * breeding round              | 1,119 | 15.88 | 0.0001   |
| Diet * sex                        | 1,121 | 8.29  | 0.005    |
| Diet * breeding round* sex        | 1,118 | 6.11  | 0.01     |
| Egg number * sex                  | 1,122 | 6.36  | 0.01     |
| Egg number * breeding round       | 1,130 | 9.47  | 0.003    |
| Egg number * sex * breeding round | 1,125 | 18.32 | < 0.0001 |
| <i>DHT concentration</i>          |       |       |          |
| Egg number                        | 1,133 | 21.81 | < 0.0001 |
| Diet                              | 1,83  | 1.58  | 0.21     |
| Breeding round                    | 1,79  | 0.69  | 0.41     |
| Sex                               | 1,136 | 1.60  | 0.21     |
| Diet * breeding round             | 1,51  | 0.90  | 0.35     |
| Sex * breeding round              | 1,137 | 2.24  | 0.14     |
| Diet * sex                        | 1,137 | 3.10  | 0.08     |
| Diet * breeding round* sex        | 1,136 | 12.63 | 0.0005   |

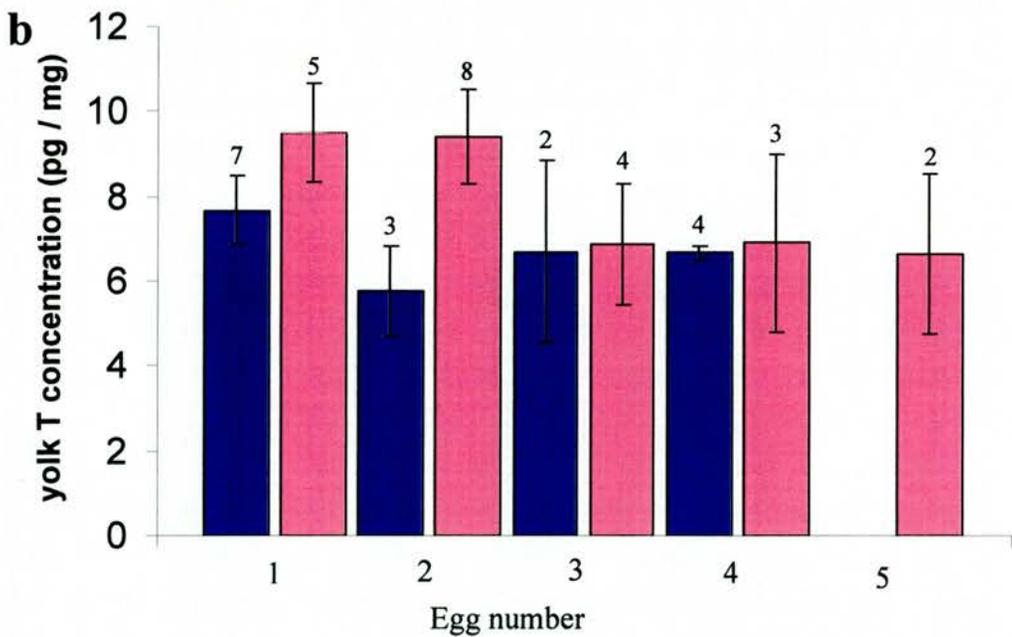
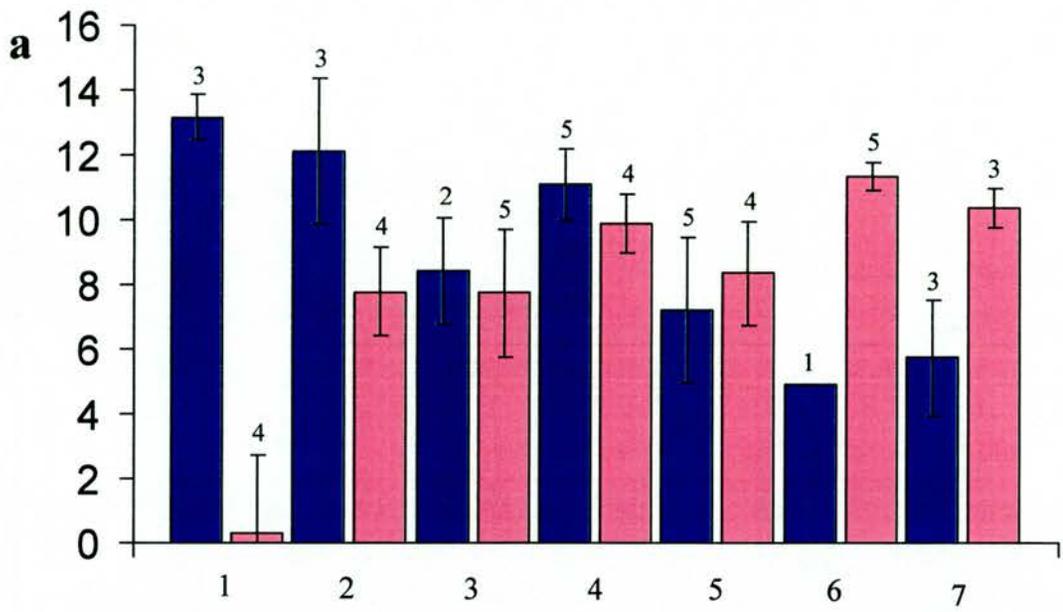


Figure 7. T concentration in male and female eggs (means  $\pm$  SE) in relation to position in the laying sequence for the 14 females that laid eggs (a) on the HQ diet in round one and (b) on the LQ diet in round two. Blue bars represent male eggs and pink bars represent female eggs. Number of eggs that were assayed are given above bars.

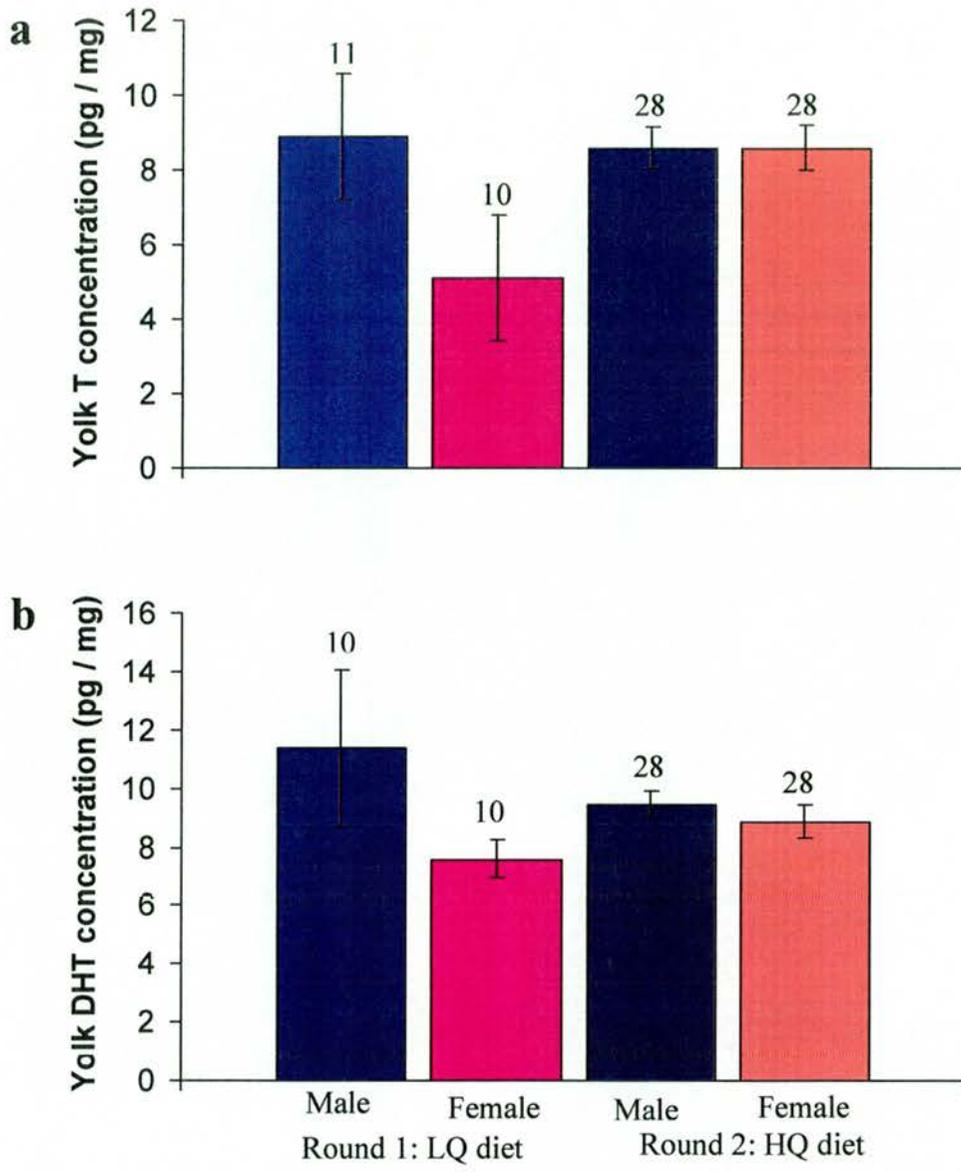


Figure 8. Concentration (means  $\pm$  SE) of (a) T and (b) DHT for male and female eggs paired for females that laid fertile clutches whilst on the LQ diet in round one and on the HQ diet in round two (n = 9). Number of eggs that were assayed are given above bars.

( $F_{1,28} = 0.03$ ,  $p = 0.86$ ). However, there was an effect of breeding round on female mass ( $F_{1,29} = 14.86$ ,  $p = 0.0006$ ). Females lost mass during the first experimental diet, i.e. prior to the first round of breeding (mean  $\pm$  SE,  $-0.16 \pm 0.29$ g) and put on mass during the second experimental diet ( $+1.57 \pm 0.29$ g). The interaction between diet and breeding round was not significant ( $F_{1,28} = 1.15$ ,  $p = 0.29$ ). In the analysis of mass change due to breeding, there was a near significant interaction between diet and breeding round ( $F_{1,28} = 4.18$ ,  $p = 0.052$ , Fig. 9a). Females that were on the LQ diet in round two lost more mass than when they bred on the HQ diet, but females that were on the LQ diet in round one lost mass to an equivalent degree to when they were on the HQ diet (Fig. 9a).

### **Male body mass**

Paired analyses were carried out on the mass change during breeding for 30 males. As with the females, there was an interaction between diet and breeding round ( $F_{1,28} = 5.94$ ,  $p = 0.02$ , Fig. 9b).

## **Discussion**

### **Levels of investment**

Females laid more and heavier eggs on the HQ diet compared with on the LQ diet. Similar results have been found previously in zebra finches (Monaghan *et al.* 1996, Selman & Houston 1996, Williams 1996a, Rutkowska & Cichon 2002, this thesis Chapter 4). There was evidence of a carry-over effect between breeding rounds, since females on the HQ diet in round one laid heavier eggs on the LQ diet in round two compared with females that were on the LQ diet in round one. This may be because females were able to store some limiting essential amino acid that was then used over a longer period (Williams 1996a) and also because females were not allowed to rear young and so may have maintained a higher body condition than if they had reared chicks (see Chapter 4).

This carry-over effect was also evident in the androgen data. In round one, eggs laid on the HQ diet that were infertile, contained higher concentrations of T than did fertile eggs. In round two this pattern was reversed, showing that females maintained their level of investment between breeding attempts. While infertility could have been a consequence of the high concentrations of T in these eggs, the lower levels of T in fertile eggs could

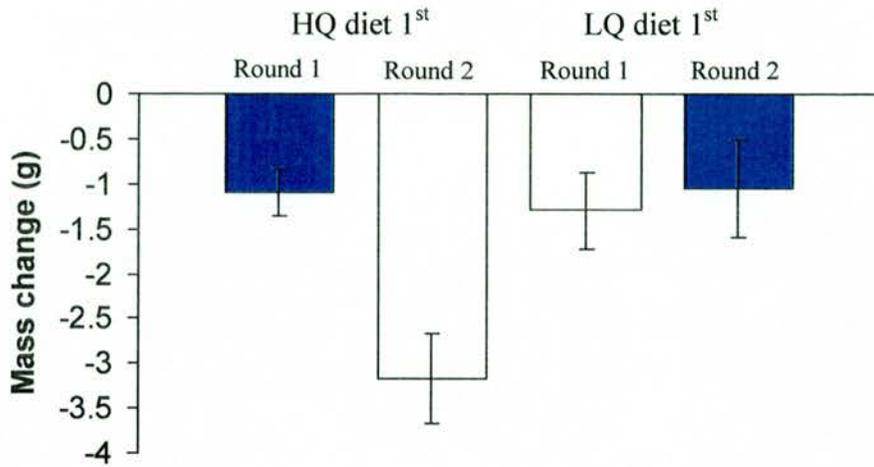


Figure 9a. Mass change (mean  $\pm$  SE) after breeding for females that laid a clutch on both diets. Seventeen females laid on the HQ diet in round one and on the LQ diet in round two, paired t-test,  $t = 13.74$ ,  $p = 0.002$ . Thirteen females laid on the LQ diet in round one and on the HQ diet in round two,  $t = 0.40$ ,  $p = 0.70$ .

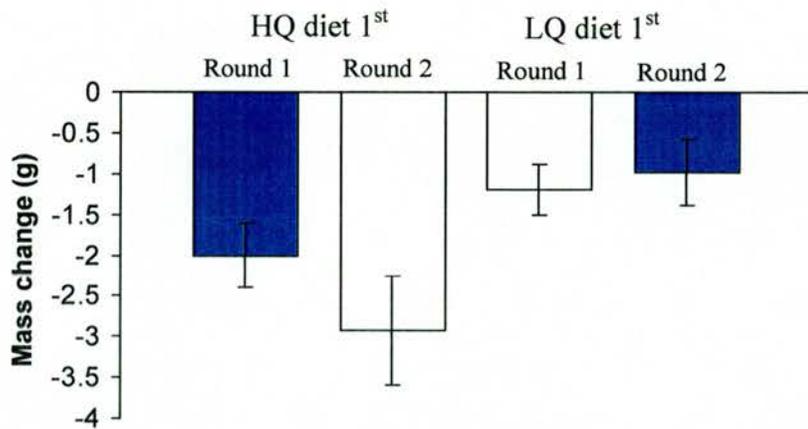


Figure 9b. Mass change (mean  $\pm$  SE) after breeding for males whose mate laid a clutch on both diets. Seventeen males incubated a clutch on the HQ diet in round one and on the LQ diet in round two, paired t-test,  $t = 1.42$ ,  $p = 0.17$ . Thirteen males incubated a clutch on the LQ diet in round one and on the HQ diet in round two,  $t = 0.43$ ,  $p = 0.77$ .

have been because embryo development causes a reduction in T. Support for the latter hypothesis comes from work on Leghorn chicken eggs by Elf & Fivizzani (2002), who found that significant declines in androgen levels were evident as early as day one of embryonic development. In the current study embryos were 3 days old, suggesting that declines in androgen levels would be detectable.

In contrast, there was no difference in T concentration between fertile and infertile eggs on the LQ diet in round one, but this may have been because infertile LQ diet eggs had initially lower concentrations of T than HQ eggs, and embryonic development (and hence T metabolism) might have been slower in these eggs (Eising *et al.* 2001). If greater T allocation is costly in some way, the higher T concentration in infertile HQ diet eggs suggests a greater investment by these females, thus providing support for the first prediction.

Females on the LQ diet might deposit lower levels of T either if doing so is costly in some way to the female, or if high levels of T are deleterious to the embryo (Gil *et al.* 1999, Sockman & Schwabl 2000, Mazuc *et al.* 2003), an effect to which 'lower quality' embryos (from smaller, less well-provisioned eggs) may be particularly vulnerable (Royle *et al.* 2001). There was no evidence in the current study that females were compensating for their lighter eggs on the LQ diet with respect to yolk androgens, as was found to be the case in a colony of black-backed gulls (Groothuis & Schwabl 2002).

### **Within-clutch patterns of egg mass and androgen deposition**

There was also a difference in the pattern of egg mass with laying sequence, as I have reported previously (Chapter 4). Egg mass increased with laying sequence on the HQ diet, but not on the LQ diet, which might represent the adoption of different adaptive strategies with respect to competitive hierarchies established by hatching asynchrony (Slagsvold *et al.* 1984). Although zebra finches hatch asynchronously in the laboratory, the degree of hatching asynchrony is reportedly less in the wild, although the youngest chicks are more likely to starve (Zann 1996). However, there was no evidence for the second prediction that concentrations of yolk androgens would also differ with position in the laying sequence on the two diets. T and DHT declined with position in the laying sequence on both diets, as has been described previously for this species (Gil *et al.* 1999).

Data collected in different field seasons, of varying food abundance, from black-headed gulls, similarly did not show variation in within-clutch patterns of androgen deposition (Eising *et al.* unpublished data, cited in Groothuis & Schwabl 2002). However, Pilz *et al.* (2003) found that older female starlings allocated more T and A<sub>4</sub> to later laid eggs but younger females did not. In red-winged blackbirds, there was variation in intra-clutch patterns of egg mass (Blank & Nolan 1983) with older females, but not yearlings, laying larger eggs towards the end of the laying sequence. In this latter species, Lipar & Ketterson (2000) also found considerable variation between females in the intra-clutch patterns of T deposition, although this was not linked to female age in their study.

In white stork chicks, *Ciconia ciconia*, the within-clutch gradient of plasma testosterone was found to vary according to female condition (Sasvari *et al.* 1999). Chicks hatched by females in poor condition had a high gradient of plasma T deposition within the brood, which increased the competitive hierarchy and facilitated brood reduction. Chicks hatched by females in good condition had a lower gradient of plasma T deposition within the brood and, consequently, there was reduced mortality. The authors suggested that plasma levels of the chicks reflected deposition of yolk androgens by the female implying, therefore, that these differences were under maternal control.

### **Differential sex allocation**

In contrast to previous findings (Chapter 4) there was no significant sex ratio bias on either diet and also no significant intra-clutch sex ratio bias. However, there were missing data in the current study due to difficulties with extracting sufficient DNA from small quantities of embryonic material. It was essential to allow embryos to develop only minimally in order to reduce the effect of embryonic development on yolk androgen concentration (see Elf & Fivizzani 2002). This means that the sex ratio analysis may be less robust.

There was, however, support for the third prediction concerning differential investment in male and female eggs with respect to yolk androgens. On the HQ diet in round one, T decreased with position in the laying sequence for male eggs, but increased with position in the laying sequence for female eggs. If females are the more expensive sex (Kilner 1998) this may be an adaptive mechanism employed when conditions are favourable, to offset the disadvantage facing females hatching at the end of the clutch. For

females on the LQ diet in round one, male eggs contained higher concentrations of DHT and T than female eggs, but when these females were on the HQ diet in round two, there was no difference in DHT or T concentration between male and female eggs. Because daughters have relatively lower reproductive potential on a LQ diet (Haywood & Perrins 1992), females may be investing less in female eggs on the LQ diet. Higher androgens in male eggs may also partly explain why males do better on a poorer diet (Bradbury & Blakey 1998, Kilner 1998). However, this cannot be the whole story, because females still fare worse than males on a poor rearing diet when eggs are laid on the same pre-breeding diet (Martins 2003).

Although no sex differences in yolk androgen levels have been found in canaries (Schwabl 1993) or in red-winged blackbirds (Lipar & Ketterson 2000), sex differences in androgen levels have been reported both in domestic chickens (Müller *et al.* 2002) and peacocks, *Pavo cristatus* (Petrie *et al.* 2001). In the latter study, hormone levels were determined after ten days incubation and therefore would be unlikely to represent maternal hormone allocation (see Elf & Fivizzani 2002). In the current study, the incubation period was minimised to only three days and so the data are more likely to reflect initial deposition by the female.

## Conclusions

In conclusion, this study suggests that maternal deposition of yolk androgens does vary according to diet quality, demonstrating a certain degree of flexibility on the part of the female in relation to her environment. There was evidence of a carry-over effect with respect to certain aspects of the egg mass and androgen data, but not others, possibly because females were reacting both to their own body condition as well as the current resources available for rearing young. Certainly, in the first round of breeding, females on the HQ diet were depositing more androgens in eggs compared with females on the LQ diet. The effect that higher androgen concentration may have on zebra finches is currently under investigation (L. Gilbert. pers. comm.), although the costs to the laying female have not yet been studied.

Females were also found to differentially allocate androgens to male and female eggs. The mechanism by which this is achieved and the effect that this has on the offspring

is not known, but it may partly explain results from studies that have found females to be disadvantaged compared to males when females have laid eggs and reared chicks on a poorer diet (Bradbury & Blakey 1998, Kilner 1998). The current study, in conjunction with others (Müller *et al.* 2002), suggests that, together with hatching asynchrony, female allocation of yolk androgens has the potential to affect within-clutch competitive hierarchies in a sex-specific manner.

## Chapter 6: The effect of diet quality on egg composition

### Introduction

#### Egg size

The size and composition of the avian egg greatly influence the embryo's development and its survival prospects after hatching. This is because, unlike mammals, where there is considerable transfer of nutrients and hormones from mother to foetus during gestation, in birds embryonic development takes place within the egg, whose characteristics are determined prior to laying (Winkler 1993).

Diet quality has been found to have a strong effect on egg mass in zebra finches, *Taeniopygia guttata* (Monaghan *et al.* 1996, Selman & Houston 1996, Williams 1996a, Rutkowska & Cichon 2002). I found previously (Chapter 4) that, not only did diet quality affect egg mass, but that it also affected the pattern of egg mass change with laying sequence. On a high quality (HQ) diet egg mass increased with position in the laying sequence whereas, on a low quality (LQ) diet egg mass tended to decrease with position in the laying sequence. These differences may be adaptive in asynchronously-hatching species, representing brood survival and brood reduction strategies respectively, by decreasing or enhancing within-clutch competitive hierarchies (Slagsvold *et al.* 1984).

In addition, I found (Chapter 4) that, on the LQ diet there was lower hatching success (also reported by Selman & Houston 1996). Chicks that did hatch from eggs laid on the LQ diet were also lighter and had shorter tarsi than chicks that hatched from eggs laid on the HQ diet, after controlling for egg mass. This suggests that the eggs laid on the LQ diet were of inferior quality.

In general, larger size confers several benefits on eggs. When eggs are not being incubated, large eggs take longer to cool than small eggs, due to their smaller surface area:volume ratio (O'Connor 1979). For the same reason, large eggs lose proportionately less water through evaporation (Drent 1970) and at a relatively lower rate (Carey *et al.* 1983). Small eggs are also more easily predated (Montevecchi 1976, Verbeek 1988). Additionally, in precocial species such as ducks, heavier hatchlings (which come from larger eggs) are better able to maintain homeothermy at low temperatures (Rhymer 1988). In my previous study (Chapter 4), there was greater post-hatching mortality of chicks that hatched from lighter eggs on both diets. Many other

avian studies have found positive relationships between egg size and subsequent offspring growth and survival (Perrins 1965, Schifferli 1973, Parsons 1970, Lundberg & Vaisanen 1979, O'Connor 1979, Heaney & Monaghan 1995), including studies that have controlled for parental quality (Nisbet 1978, Ricklefs 1984, Amundsen & Stockland 1990, Bolton 1991, Magrath 1992). However, in most cases, the influence of egg size on chick growth is limited to early on in the nestling period (Smith & Bruun 1998) especially in altricial young, where parental quality plays a greater role in determining chick size (Carey 1996). Furthermore, fitness benefits may be more apparent when conditions are harsh (Styrsky *et al.* 1999).

### **Egg macro-composition**

As well as egg size, it is important to consider egg composition. Higher quality individuals are predicted to invest more resources in reproduction than are low quality individuals (Pilz *et al.* 2003), and this may be reflected in egg quality as well as egg size. Both albumen and yolk are composed principally of water (Carey 1996). In addition, they also comprise carbohydrate, lipid, protein and inorganic ions, in varying proportions depending on the species. Albumen comprises the major proportion of the egg, and contains on average 46% and 71% of the total egg protein in precocial and altricial species respectively (Carey *et al.* 1980). Albumen limits the structural growth of the chick and is entirely used up by the time the chick hatches (Romanoff & Romanoff 1949, Freeman & Vince 1974), whereas yolk, which is high in lipids, serves as the primary source of energy for the developing young (Hill 1993).

Allometric analyses are typically used to examine the relation between egg size and egg composition (Reiss 1989). The log of egg component mass is regressed on the log of egg mass, and the slope describes the rate at which the egg component varies with egg size. A slope equal to 1.0 (isometry) indicates that increases in egg size are accompanied by proportionate increases in the particular egg component; consequently, the percentage of that egg component will remain constant across eggs of different sizes. A slope of greater than 1.0 (positive allometry) indicates that the component mass increases disproportionately with increases in egg mass, so that large eggs will contain proportionately more component mass than will smaller eggs. Conversely, a slope of less than 1.0 (negative allometry) demonstrates that a component increases proportionately less than egg mass, so that large eggs will have relatively smaller amounts of that egg component.

Egg composition varies both between and within species. Interspecies differences have been correlated with various levels of hatching precocity among taxa (Carey *et al.* 1980, Sotherland & Rahn 1987). Eggs of precocial species tend to have a higher proportion of yolk in the egg and a corresponding lower proportion of albumen. This is thought to reflect the greater physiological and behavioural maturity and yolk reserves of precocial hatchlings (Carey *et al.* 1980, Sotherland & Rahn 1987). Allometric patterns of variation in egg composition also differ between precocial and altricial species (Hill 1995). The general pattern in altricial birds is that of positive allometry for wet albumen, and, negative allometry for wet and dry yolk. In precocial species, however, wet and dry yolk and albumen mass tend to increase isometrically with egg size (Hill 1995).

Within a species there may also be considerable variation in egg composition (Williams 1994), reflecting differences in territory quality (Arnold 1992), food availability (Hochachka 1988, Arnold *et al.* 1991) and female body condition (Alisaukas 1986). Although it is known that egg composition influences chick growth and survival as well as egg size (Ricklefs 1984, Nager *et al.* 2000b), changes in size have not been generally separated from changes in nutrient content, and there has been little research on the interrelationships among egg size, egg composition, and offspring fitness (Hepp *et al.* 1987, Bernardo 1996). The trade-off between egg number and egg quality was investigated in an experimental study in lesser black-backed gulls, *Larus fuscus*. Females were induced to lay additional eggs, which were examined with respect to both their composition and the probability that they would produce a fledgling, in relation to egg size (Nager *et al.* 2000b). Chicks from eggs laid later in the laying sequence were found to have a lower survival probability, and these eggs had a relatively lower lipid content and a relatively greater water content.

However, there has been a lack of consensus concerning which component of the egg has the most important effect on chick development, i.e., as to whether it is yolk (Kear 1965, Parsons 1970), albumen (Nisbet 1978) or water content (Finkler *et al.* 1998) that is most important in determining nestling quality. The answer may depend on the species. In species with intense sibling competition, it would be advantageous for a chick to be structurally larger at hatching whereas, in species that lay only one egg, there would be less selection for increased size of the hatchling (Birkhead & Nettleship 1984). Therefore, it is thought that in altricial species with brood reduction, where chick skeletal size is important, females ought to invest more in the protein

content of eggs (principally in albumen in altricial species) with increasing egg size (Boersma 1982). Conversely, if reserves at hatching are more important, such as in most altricials, then females ought to invest more in the lipid (yolk) component of larger-sized eggs (Birkhead & Nettleship 1984).

Advances have been made in this field with the implementation of allometric engineering, which involves directly altering the composition of eggs. By experimental removal of part of the yolk, the albumen or both components from leghorn chicken, *Gallus gallus domesticus*, eggs, Finkler *et al.* (1998) demonstrated that the water content of albumen was the primary determinant of hatchling tibiotarsus length and body mass. Modification of the amount of yolk did not influence hatchling mass or size.

Other potentially important components of eggs include carotenoids, immunoglobulins and androgens and, it is known that females can differentially invest in eggs with respect to these components (Bryant & Tatner 1990, Schwabl 1993, 1997, Heeb *et al.* 1998, Gil *et al.* 1999, Royle *et al.* 1999, 2001, Blount *et al.* 2002a). Such maternal effects are thought to be adaptive in preparing the chick for the local environment in which it will grow (Bernardo 1996, Mousseau & Fox 1998).

### **Carotenoids**

Carotenoids are lipid-soluble hydrocarbons, which give yolks their characteristic yellow colour (Blount *et al.* 2002a). They are thought to be important because of their function in the deactivation of reactive oxidative metabolites and free radicals, which are by-products of normal metabolism and immune defence and, which can cause extensive DNA, protein, and lipid damage (von Schantz *et al.* 1999). Oxidative stress could be particularly prominent in developing embryos because of the high rates of oxidative metabolism during growth (Blount *et al.* 2000). Carotenoid levels are determined by their availability in the mother's diet, as they cannot be synthesised or stored for long periods (Surai *et al.* 1998, Blount *et al.* 2000, 2002a), although there is evidence suggesting that birds can metabolise certain carotenoids from other carotenoid precursors (McGraw *et al.* 2002). Carotenoids are especially limited in the avian embryo - a closed system, which relies entirely on maternally deposited resources (Hörak *et al.* 2002).

That carotenoid availability in the diet influences antioxidant level in the egg was experimentally demonstrated in a supplementary feeding experiment on lesser

black-backed gulls (Blount *et al.* 2002a). The plasma of carotenoid-fed females had significantly higher carotenoid concentrations, which was subsequently transferred into the eggs that they laid. The eggs of these females therefore contained significantly more carotenoids than controls and, were consequently associated with higher antioxidant activity. Because females need carotenoids for their own use, and because they are a limited resource, there is potentially a trade-off in carotenoid allocation between maternal and offspring fitness (Blount *et al.* 2000). Female barn swallows, *Hirundo rustica*, that were immunologically-challenged with an antigen before egg laying produced eggs with smaller concentrations of lutein (the main carotenoid in barn swallows) compared with control females (Saino *et al.* 2002).

Females may differentially invest in their clutch according to the perceived value of a mating attempt with respect to, for example, clutch size (Petrie & Williams 1993), egg size (Cunningham & Russell 2000), and testosterone (T) levels (Gil *et al.* 1999). The same logic should also apply to carotenoid deposition, and this was investigated in barn swallows (Saino *et al.* 2002). Tail length, a sexually selected trait, was artificially lengthened or shortened. Females were predicted to deposit more carotenoids in eggs fathered by more attractive males (those with longer tails). Contrary to predictions, mates of tail-shortened males were found to deposit higher lutein concentrations in their eggs compared with females mated to controls or tail-elongated males. This was interpreted as adaptive since the offspring of short-tailed males have larger exposure and/or susceptibility to parasites (Møller 1994). Therefore, females invested according to the perceived vulnerability of the offspring, rather than according to the value of such offspring.

Female zebra finches deposit higher concentrations of androgens in eggs when laying on a HQ diet compared with on a LQ diet (Chapter 5). This might be beneficial since T has been shown to accelerate embryonic growth in black-headed gulls, *Larus ridibundus* (Eising *et al.* 2001) and to increase begging and growth rates post-hatching in canaries *Serinus canaria* (Schwabl 1993, 1996a). However, T also has immunosuppressive effects (Ketterson & Nolan 1999). T was found to reduce nestling growth and survival rates in American kestrels, *Falco sparverius* (Sockman & Schwabl 2000) and was correlated with low hatching success in house sparrows, *Passer domesticus* (Mazuc *et al.* 2003) and in zebra finches (this thesis Chapter 3). Therefore, carotenoids may have an important protective role, with respect to yolk T levels. This was found to be the case in lesser black-backed gulls. The amount of yolk T was found to increase

with laying sequence, whereas carotenoids decreased with laying sequence and was particularly low when food was scarce. Under these conditions last-laid eggs fared poorly, and this facilitated rapid brood reduction (Royle *et al.* 2001).

## **Aims**

It has been suggested that females may facultatively adjust egg composition to increase their own reproductive success (Alisauskus 1986, Hepp *et al.* 1987). The aim of this chapter was to examine egg composition in relation to diet quality, with particular interest in intra-clutch differences. Within clutches, larger eggs laid under both diet regimes were predicted to contain a higher proportion of wet albumen, since larger hatchlings (Finkler *et al.* 1998) would be at a competitive advantage relative to their siblings. Given the difference in egg mass with laying sequence found previously on the two diets (Chapters 4 & 5), on the HQ diet, this would mean that later hatching chicks would be better able to compete with their older, larger siblings. On the LQ diet, this would mean that the early-hatching chicks would be further advantaged, and would rapidly out-compete their younger siblings. Carotenoids were predicted to follow the same pattern, if females were allocating carotenoids according to the perceived value of the offspring. Alternatively, because yolk T, however, was found to decrease with position in the laying sequence on both the HQ and the LQ diet (Chapter 5), given their potentially protective role, carotenoids might also decrease with laying sequence on both diets.

## **Methods**

### **Experimental design**

Thirty-two female zebra finches were weighed and randomly assigned to one of two diet groups for six weeks. Females on the HQ regime were given foreign finch mix *ad libitum* (approx. 14% protein), supplemented daily with Haith's egg biscuit (13.2% protein), mixed with hard-boiled hen's egg (13.5% protein), PBX protein crumb (26% protein) and fresh spinach. Females on the LQ regime were given foreign finch mix *ad libitum*, and spinach three times a week. Both groups had access to cuttlefish bone and fresh water *ad libitum*.

After six weeks females were re-weighed and paired randomly with males with whom they had not previously bred. The pairs were placed in individual breeding

cages, equipped with open nest boxes and nesting material. For the six weeks prior to pairing, all males had been caged in single sex groups in a separate room on the standard laboratory diet of foreign finch mix, supplemented with spinach and egg mix once a week. Once paired, males were then kept on the same diet as their mate and these diets were maintained during egg laying and incubation.

Nests were checked every morning for eggs. Newly laid eggs were removed, weighed to the nearest 0.01g on an electronic balance and numbered. Eggs were replaced with dummy eggs to maintain the clutch size.

The yolk and albumen components of the eggs were separated in a small petri dish. The yolk was scored visually for carotenoid content using a Roche yolk colour fan, on a scale of 1 –15, with 15 being the darkest shade of yellow. The albumen and yolk were weighed to the nearest 0.0001g to obtain their wet mass, dried to constant weight at 65°C for 48 hours, and re-weighed to obtain their dry weight.

### **Statistical analyses**

The allometry of egg composition was investigated by regression of the logarithm of each component's mass on the logarithm of fresh egg mass. Analyses were carried out using a repeated measures mixed general linear model (GLM) procedure, with female as a random factor, egg number as a covariate and diet as a fixed factor, as described in more detail in Chapter 2.

## **Results**

### **Clutch size and egg mass**

There was no difference in the proportion of females that laid eggs on the two diets. Clutches were laid by 14/16 HQ females and by 15/16 LQ females ( $G = 0.36$ ,  $df = 1$ ,  $p > 0.5$ ). HQ females began laying a significantly shorter time after pairing than LQ females (median days between pairing and laying of the first egg (interquartile range), HQ: 3(1 – 7), LQ: 7 (4 – 9), Mann - Whitney U,  $W = 163.0$ ,  $p = 0.04$ ). HQ females also laid significantly larger clutches (median (IQR), HQ: 6 (5.75 – 7), LQ: 4 (2 – 5),  $W = 307.5$ ,  $p < 0.001$ ). Mean egg mass per clutch was also significantly greater for HQ females (mean  $\pm$  SE, HQ:  $1.30 \pm 0.05$ g, LQ:  $1.04 \pm 0.03$ g, independent sample t test,  $t = 5.11$ ,  $df = 27$ ,  $p < 0.001$ ).

In a repeated measures GLM, there was a near significant interaction between egg mass and position in the laying sequence ( $F_{1,128} = 3.40$ ,  $p = 0.07$ , Fig. 1). Egg mass increased significantly with laying sequence on the HQ diet ( $F_{1,82} = 15.25$ ,  $p = 0.0002$ ), but there was no effect of laying sequence on egg mass on the LQ diet ( $F_{1,50} = 1.04$ ,  $p = 0.31$ ). Egg mass was not affected by female body mass on either diet ( $F_{1,31} = 0.66$ ,  $p = 0.42$ ).

### Macro-composition of eggs

Both wet and dry log yolk and albumen mass were highly correlated with log egg mass in both diet groups ( $p < 0.001$  in all cases, Table 1). Therefore, heavier eggs contained absolute greater quantities of wet and dry, yolk and albumen. On average, HQ diet eggs comprised (mean  $\pm$  SE)  $56.52 \pm 0.60\%$  wet albumen and  $16.69 \pm 0.26\%$  wet yolk. The respective figures for the LQ diet eggs were  $53.85 \pm 0.85\%$  and  $17.20 \pm 0.44\%$ . The remaining percentage comprised eggshell and small amounts of albumen and yolk that adhered to the shell and pipette tip. HQ eggs comprised  $6.21 \pm 0.08\%$  dry albumen and  $6.93 \pm 0.12\%$  dry yolk. The respective figures for the LQ eggs were  $5.66 \pm 0.09\%$  and  $7.29 \pm 0.20\%$ . Therefore, the water component of albumen made up by far the largest single proportion of the egg ( $50.31 \pm 0.53\%$  in HQ diet eggs,  $48.19 \pm 0.91\%$  in LQ diet eggs).

Allometric analyses revealed that for both diets, the slopes of log wet and dry yolk mass on log egg mass did not differ significantly from 1.0 (isometry), and therefore larger eggs contained proportionately similar amounts (although absolutely larger amounts) of yolk as did smaller eggs (Table 1). In a GLM, diet had no significant effect on the proportion of wet yolk ( $F_{1,42} = 0.05$ ,  $p = 0.83$ ) or dry yolk ( $F_{1,45} = 0.13$ ,  $p = 0.72$ ). Laying sequence also had no significant effect on the proportion of wet yolk ( $F_{1,73} = 0.01$ ,  $p = 0.91$ ), or dry yolk ( $F_{1,74} = 0.17$ ,  $p = 0.68$ ).

For both diets, the slope of log dry albumen on log egg mass did not differ significantly from 1.0, again indicating that larger eggs contained proportionately similar amounts to smaller eggs (Table 1). Again, in a GLM, diet had no significant effect on the proportion of dry albumen ( $F_{1,42} = 1.62$ ,  $p = 0.21$ ), but the proportion of dry albumen increased significantly with laying sequence when data from both diets were combined ( $F_{1,70} = 4.90$ ,  $p = 0.03$ ). The effect of laying sequence was not significant in a separate analysis for HQ diet eggs only ( $F_{1,31} = 1.57$ ,  $p = 0.22$ ) or for LQ diet eggs only ( $F_{1,33} = 2.92$ ,  $p = 0.10$ ).

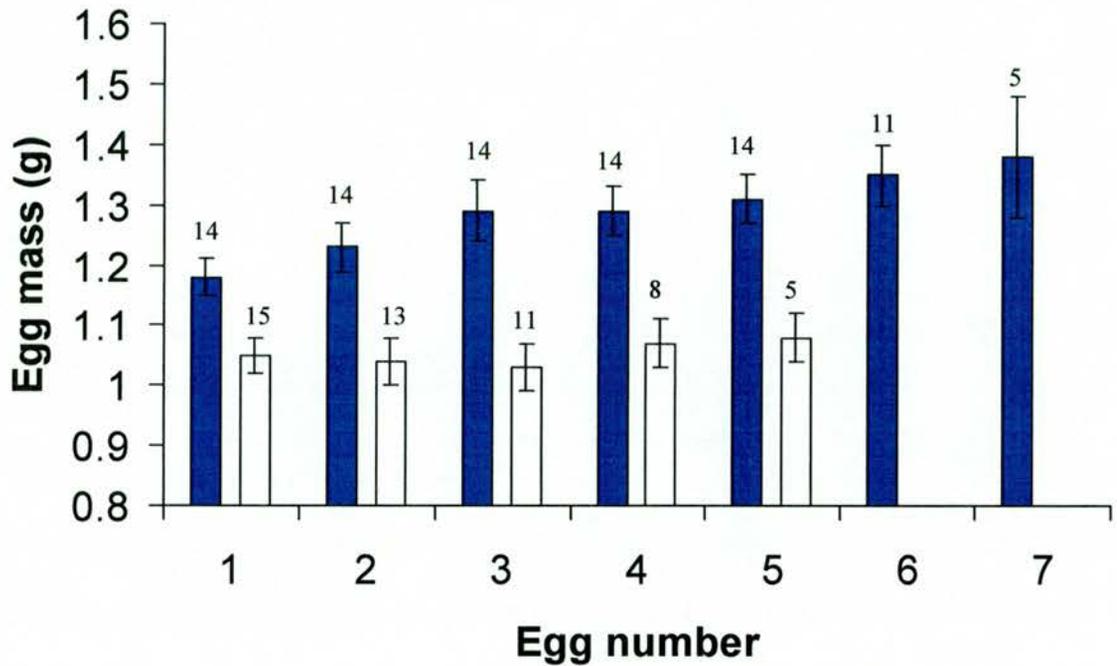


Figure 1. Change in egg mass with position in the laying sequence, showing means  $\pm$  S.E for eggs laid by females on the HQ (n = 14) and LQ (n = 15) diets. Blue bars represent eggs laid by females on the HQ diet and white bars represent eggs laid by females on the LQ diet. Numbers of eggs are given above bars.

Table 1. Slopes and 95% confidence intervals (C.I.) for log wet and dry masses of yolk and albumen for HQ and LQ diets. Asterisks indicate slopes that differed significantly from 1.0.

| Diet      | Egg component    | Slope $\pm$ 95% C.I. | F                          | p        |
|-----------|------------------|----------------------|----------------------------|----------|
| <b>HQ</b> | wet yolk mass    | 0.85 $\pm$ 0.30      | F <sub>1,35</sub> = 33.19  | < 0.0001 |
|           | dry yolk mass    | 0.88 $\pm$ 0.32      | F <sub>1,35</sub> = 31.50  | < 0.0001 |
|           | wet albumen mass | 1.31 $\pm$ 0.19 *    | F <sub>1,24</sub> = 210.56 | < 0.0001 |
|           | dry albumen mass | 1.20 $\pm$ 0.27      | F <sub>1,27</sub> = 84.16  | < 0.0001 |
| <b>LQ</b> | wet yolk mass    | 0.86 $\pm$ 0.54      | F <sub>1,41</sub> = 10.31  | 0.0003   |
|           | dry yolk mass    | 0.99 $\pm$ 0.55      | F <sub>1,35</sub> = 13.29  | 0.0009   |
|           | wet albumen mass | 1.75 $\pm$ 0.31*     | F <sub>1,39</sub> = 132.86 | < 0.0001 |
|           | dry albumen mass | 1.13 $\pm$ 0.31      | F <sub>1,38</sub> = 58.82  | < 0.0001 |

On both the HQ and LQ diets, the slopes of log wet albumen on log egg mass were significantly greater than 1.0 (positive allometry), indicating that heavier eggs contained proportionately greater amounts of wet albumen than lighter eggs. There was a significant interaction between log egg mass and diet ( $F_{1,73} = 7.08$ ,  $p = 0.01$ , Fig. 2), due to lighter LQ diet eggs containing smaller amounts of wet albumen.

### **Carotenoid levels**

Carotenoid levels, as indicated by Roche colour scores, decreased significantly with laying sequence on both diets, but the rate of decline was significantly greater on the LQ diet (interaction between diet and laying sequence:  $F_{1,93} = 6.96$ ,  $p = 0.01$ , Fig. 3). In separate analyses for the HQ diet and LQ diet eggs, the mean rate of decline ( $\pm$  SE) in colour for HQ eggs was  $-0.22 \pm 0.07$  ( $F_{1,62} = 4.80$ ,  $p = 0.03$ ), compared with  $-0.69 \pm 0.14$  for LQ diet eggs ( $F_{1,47} = 22.17$ ,  $p < 0.0001$ ). Carotenoid concentration was not significantly affected by wet yolk mass ( $F_{1,110} = 0.18$ ,  $p = 0.67$ ) and the interaction between wet yolk mass and diet was not significant ( $F_{1,117} = 2.80$ ,  $p = 0.10$ ).

### **Female mass**

There was no difference in female mass between the two groups prior to the experimental diets (mean  $\pm$  SE, HQ females ( $n = 32$ ):  $19.35 \pm 0.48$ g, LQ females ( $n = 32$ ):  $19.11 \pm 0.66$ g, independent samples t-test,  $t = 0.29$ ,  $df = 30$ ,  $p = 0.77$ ). There was no difference in the percentage mass change after six weeks on the diets and prior to pairing (HQ females:  $-0.18 \pm 1.7\%$ , LQ females:  $-3.10 \pm 2.2\%$ ,  $t = 1.06$ ,  $df = 30$ ,  $p = 0.30$ ). After laying, females that laid eggs lost mass to an equivalent degree (HQ females ( $n = 14$ ):  $-8.96 \pm 1.6\%$ , LQ females ( $n = 15$ ):  $-10.66 \pm 1.4\%$ ,  $t = 0.80$ ,  $df = 27$ ,  $p = 0.43$ ).

### **Male mass**

There was no difference in the mass of males in the two diet groups prior to pairing ( $t = 1.07$ ,  $df = 30$ ,  $p = 0.29$ ). Following incubation, males in both groups lost mass to an equivalent degree (mean  $\pm$  SE, HQ males ( $n = 14$ ):  $-9.31 \pm 2.1\%$ , LQ males ( $n = 15$ ):  $-10.61 \pm 1.4\%$ ,  $t = 0.53$ ,  $df = 27$ ,  $p = 0.60$ ).

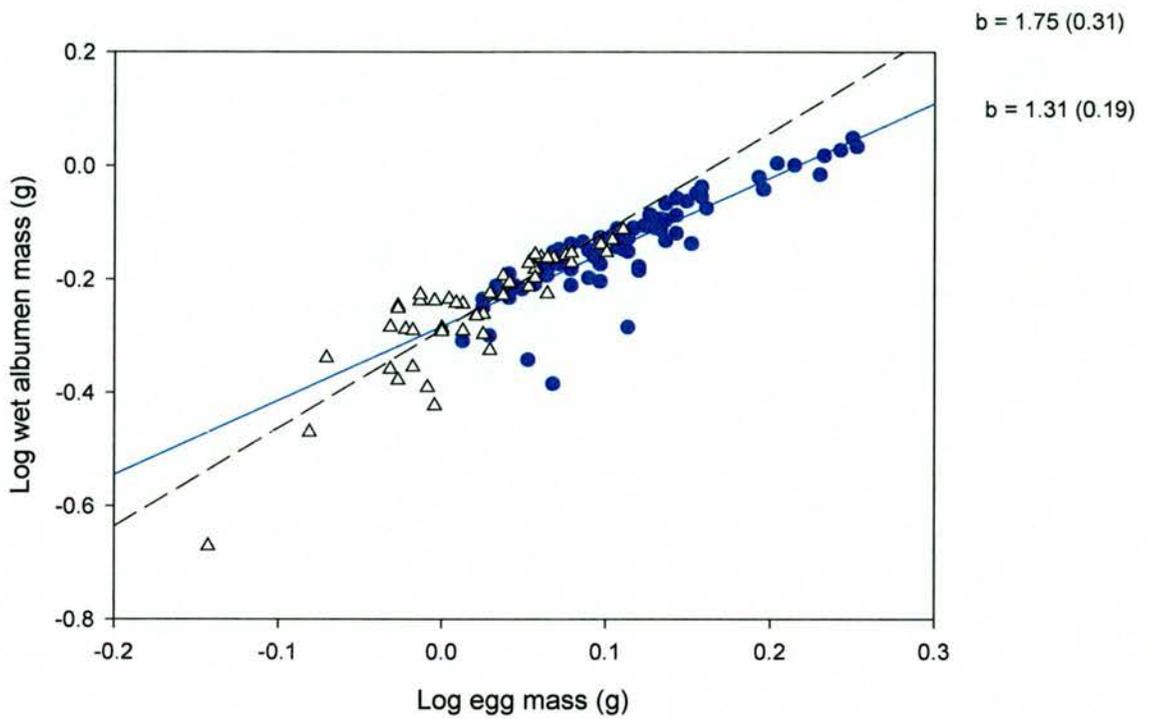


Figure 2. Allometric relationship between log wet albumen mass and log egg mass for eggs laid on HQ and LQ diets. Slopes of regression lines (95% confidence intervals) are given. Eggs of females on the HQ diet are shown by blue circles and the blue, solid regression line. Eggs of females on the LQ diet are shown by white triangles and the black, dashed regression line.

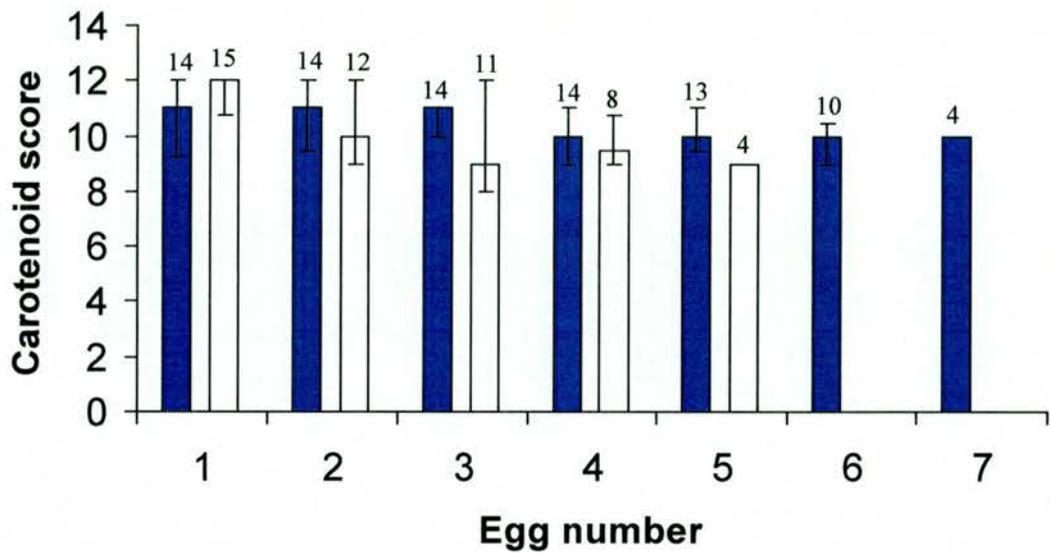


Figure 3. Darkness of yolk pigmentation (median and interquartile range) in relation to laying sequence for eggs laid on the HQ (shown by blue bars) and LQ diet (shown by white bars). The yolk colour was scored using a Roche colour fan, which ranked pigment colour from 1 (the lightest) to 15 (the darkest). Number of eggs are given above bars.

## Discussion

### Egg mass and macro-composition

The clutch sizes and the pattern of egg size variation of the birds in this experiment were qualitatively the same as those of two previous experiments on diet quality (Chapter 4 & 5): in all experiments females on the HQ diet laid larger clutches comprising heavier eggs. In addition, egg mass increased with laying sequence on the HQ diet in all experiments but not on the LQ diet.

The current study found that on both the HQ and LQ diets, heavier eggs were better provisioned with wet and dry yolk, and dry albumen (in absolute terms) and wet albumen (both absolutely and proportionately). The percentage composition of yolk was slightly lower than that reported for altricial species in general (Carey 1996), including zebra finches. Lill & Fell (1990) reported that yolk comprised 26% of total egg mass, about 10% greater than that found in the current study. Many earlier studies focused on yolk as the important constituent in determination of hatchling quality, particularly its lipid component (Ankney 1980, Alisaukas 1986), although there was later interest in the non-lipid fraction of the egg yolk (Duncan 1988).

These early studies dismissed the water component as having any importance in determining hatchling quality (Arnold *et al.* 1991, Carey 1986). Despite this, many researchers found that variation in egg size was primarily due to differences in water content (Ricklefs 1984, Arnold 1989): annual variation in egg composition was found to be primarily due to wet component masses (Birkhead & Nettleship 1984, Meathrel *et al.* 1987), as was variation due to food supplementation (Hochachka 1988). However, increases in water content were generally considered non-adaptive (Hochachka 1988), except in the case of sea birds, where it was thought to have an excretory purpose to deal with the high salt content of the chick's invertebrate diet (Ricklefs *et al.* 1978). Hill (2003) removed a proportion of the wet albumen and found that it decreased chick size at hatch, although they had similar growth to controls after 20 days *ad libitum* access to food. The author attributed this effect on hatchling size to the protein component of albumen and not the water component. However, Finkler *et al.* (1998), using similar allometric engineering techniques on chicken eggs, discovered that it was specifically the water component of albumen that was largely responsible for hatchling size.

In the current study, wet albumen increased disproportionately with increasing egg mass, and this positive allometry of wet albumen is typical of altricial species (Hill 1995). However, the slope was significantly greater on the LQ diet and it can be seen (Fig. 2) that this was due to the disproportionately small quantity of wet albumen in the lighter eggs on the LQ diet. According to Finkler *et al.* (1998) since the water component of albumen is principally responsible for determining hatchling size, larger eggs that contain relatively more wet albumen should produce larger nestlings. This lends support to the adaptive hypothesis of egg mass change with laying sequence (Slagsvold *et al.* 1984), if heavier eggs produce larger, more competitive hatchlings. Having said this, there was not a strong relationship between egg mass and tarsus length at hatching (Chapter 4), unlike the relationship between egg mass and hatching mass.

### **Carotenoid content**

Assessment of the colour of the yolks was a relatively crude method by which to assess carotenoid concentration compared to more precise techniques available such as high-performance liquid chromatography (Surai *et al.* 2001). However, clear differences in yolk colour with laying sequence were visible using this colour scoring technique. Carotenoid concentration decreased with laying sequence on both diets, but on the LQ diet, the decrease was much more pronounced, and egg one on the LQ diet had a higher carotenoid concentration than egg one on the HQ diet (Fig. 3). Although the amount of carotenoid in the diets is unknown, the interaction between colour and laying sequence implied that allocation of carotenoids may be adaptive rather than a consequence of females simply depositing less and less of a limited resource with laying sequence. Differences in carotenoid concentration within the clutch might reflect differences in offspring value: the investment of more carotenoids in early laid eggs on the LQ diet might ensure the survival of the higher quality, early-hatching chicks, at the expense of later-hatching chicks, and so facilitate brood reduction. This was the case in great tits, *Parus major*, in a comparative study between rural and urban populations (Hörak *et al.* 2002). The carotenoid concentration in eggs laid by females in rural areas, but not in urban areas, declined with laying sequence, and this was associated with greater brood reduction in rural areas.

Alternatively, eggs laid early in the laying sequence have a greater need for additional carotenoids (Saino *et al.* 2002). Androgen concentration was found to

decrease with laying sequence on both the HQ and LQ diets (Chapter 5). Extra carotenoids in eggs laid at the start of the laying sequence may serve a protective role given that they are a limited resource. In lesser black-backed gulls, Royle *et al.* (2001) found that the within-clutch variation in yolk testosterone was opposite to that of yolk antioxidant concentration. The authors proposed that when resources were plentiful, eggs contained more carotenoids, and last-laid eggs would benefit from increased T, but when resources were scarce, low carotenoid and high T levels would lead to increased mortality of chicks from last eggs. Further work is needed before assessing how adjustable carotenoid and testosterone levels are within zebra finch clutches according to prevailing conditions.

One aspect of differential allocation of macro-composition and carotenoids within the clutch not investigated here was possible sex differences in egg composition. Many studies have shown that female zebra finches are more vulnerable to nutritional stress than males (de Kogel 1997, Kilner 1998, Bradbury & Blakey 1998, Birkhead *et al.* 1999, Martins 2003). In other species, differential sex allocation at the level of the eggs has been found in relation to egg size (Mead *et al.* 1987, Anderson *et al.* 1997, Cordero *et al.* 2000, 2001), and steroid hormones (Petrie *et al.* 2001). I found previously (Chapter 5) that there was an interaction between sex and laying sequence on the HQ diet with respect to androgen levels: T decreased with laying sequence in male eggs, but increased with laying sequence in female eggs. I also found that on the LQ diet, male eggs contained higher concentrations of T and DHT than female eggs. An important possibility that has yet to be examined is that of sex differences in egg composition, and differences in sensitivities to egg composition by male and female offspring. Rutkowska & Cichon (2002) found that male zebra finches were heavier at hatch than females (although I have not found this to be the case; Chapter 4) but that there were no differences in male and female egg mass. The authors suggested that this could be due to differential provisioning of eggs. In domestic chickens albumen removal was found to have longer-lasting effects on the growth rates of females compared with males (Hill 1993). Whether this could apply to other species has yet to be investigated.

In conclusion, heavier eggs were found to be better provisioned, and as such were higher quality eggs. This may partly explain differences in hatching success and hatchling quality found on the two diets (Chapter 4). Other factors may also be

important, such as androgen levels (see Chapter 5) and difference in the thickness of egg shell (Carey 1996), although the latter was not investigated in this study.

Larger eggs, as well as containing larger absolute quantities of yolk and albumen, also contained relatively more wet albumen, and, since the water content of albumen is known to affect skeletal size in chicks (Finkler *et al.* 1998), this may have important fitness consequences. The size of an egg in the laying sequence relative to the other eggs in the clutch may have a strong effect on its competitive ability and further supports the hypothesis that females are strategically adapting their laying sequence in a typical reductionalist / survivalist manner.

## Chapter 7: General discussion

This thesis has examined female reproductive investment in relation to mate attractiveness and diet quality in zebra finches, *Taeniopygia guttata*. The principal focus was on maternal effects and primary reproductive effort, but development of offspring under different experimental treatments was also investigated.

### Mate attractiveness

In Chapter 3 I found evidence of differential allocation at laying in relation to male attractiveness. In the first round of breeding, females mated to attractive males laid heavier eggs, and there was a positive relationship between female body mass and egg mass, such that heavier females laid heavier eggs. For females mated to unattractive males, heavier females did not lay heavier eggs. Egg mass is important as a target for resource allocation, since chicks hatching from heavier eggs had higher survival prospects, as has also been found to be the case in many other species (reviewed by Williams 1994). However, there was no difference in the number or quality of offspring between treatments, which may have been partly due to the unexpectedly high rate of embryo mortality among heavier eggs laid by females mated to attractive males.

Contrary to predictions that females should invest more in sons when mated to an attractive male, there was no evidence of differential investment in male and female offspring with respect to egg size or yolk androgen levels. The primary sex ratio also did not differ between treatments, in agreement with a recent study by Zann & Runicman (2003). Burley specifically suggested that the observed secondary sex ratio bias in her experiments (Burley 1981, 1986b) was brought about by differential mortality post-hatching. However, I found no evidence to suggest that this might be the case. Sex ratio skews were present at hatching due to differential mortality of male and female embryos, but in round one, this resulted in a bias in the opposite direction to that predicted.

One consideration with a cage design such as this, in which there was no free mate choice, is that there is limited opportunity for comparison of potential mates and, in addition, there may be little perceived benefit to producing attractive sons. Differences between the results of the two studies could also have been due to assortative mating in

Burley's studies, where attractive males may have acquired superior females as mates. Females of superior quality could have reared high quality offspring, but it is not clear by what mechanism this could have resulted in a sex ratio bias at fledging in the direction observed by Burley (1981, 1986b). The hypothesis that assortative mating may have been responsible for Burley's striking results requires testing. Both mate choice and reproductive effort should be investigated in aviary set-ups with assortative mating (with red- and green-ringed males in the same aviary, as was the case in Burley's set-up) and without assortative mating (where red- and green-ringed males are in separate aviaries, as described in the pilot study given in the appendix to Chapter 3).

### **Diet quality**

In Chapters 4, 5 and 6 females laid heavier eggs and larger clutches on the high quality (HQ) diet. This reflected the importance of dietary protein in egg formation in this species (Houston *et al.* 1995, Williams 1996a). Differences could also have resulted from maternal differential allocation, with females on the low quality (LQ) diet reducing their effort in response to the unfavourable conditions and elevated costs of reproduction (Hochachka 1992).

In all diet experiments, there was a difference in the intra-clutch pattern of egg mass variation. Egg mass increased with position in the laying sequence on the HQ diet, but not on the LQ diet. Since the diets were given to females six weeks prior to laying, the increase in egg mass found on the HQ diet cannot simply reflect food availability during laying (Nilsson & Svensson 1993, Murphy 1994a). Females may be employing an adaptive brood survival mechanism to offset the disadvantage to later eggs of hatching last (Slagsvold *et al.* 1984).

In contrast to the mate attractiveness experiment (Chapter 3), there was evidence of primary sex ratio manipulation in Chapter 4. In line with predictions from sex allocation theory (Trivers & Willard 1973, Charnov 1982) the sex ratio tended to be female-biased on the HQ diet, and was significantly male-biased on the LQ diet. This supports previous zebra finch studies that have found a male bias on a poor diet at hatching (Bradbury & Blakey 1998, Kilner 1998) and a recent study that has demonstrated that female zebra finches produce more male eggs as the diet deteriorates (Rutkowska & Cichon 2002). The

precise cue for this sex ratio manipulation could be a combination of the laying female's body condition, the quality of the eggs, and the expected rearing environment of the chicks. The experiment by Rutkowska & Cichon (2002) involved switching HQ or LQ diets the day after females had laid their first egg, and found that more male eggs were produced on the new LQ diet. This strongly suggests that resource availability in the eggs is an important factor, but it does not exclude the other hypotheses, although female condition would not be expected to change so rapidly in response to diet.

There was also evidence of sex ratio adjustment within the laying sequence. On the HQ diet female eggs tended to be laid later in the laying sequence, whereas on the LQ diet female eggs tended to be laid earlier in the laying sequence. Kilner (1998) found that female eggs were laid earlier in the laying sequence on both restricted and abundant food regimes, and suggested that early hatching positions were beneficial to females. In my study, on the HQ diet later eggs were heavier and had higher post-hatching survival, suggesting that females hatching from these eggs would have greater fitness.

This difference in sex ratio adjustment between the mate attractiveness and the diet quality studies may be principally because of different selection pressures (Komdeur & Pen 2002). It is generally thought that female condition may be a stronger selection pressure and this is why so few studies on a number of species have failed to find an effect of male quality on offspring sex ratio (Westerdahl *et al.* 1997, Saino *et al.* 1999, Grindstaff *et al.* 2001). Female zebra finches have been shown to be more vulnerable than males to nutritional stress (de Kogel 1997, Kilner 1998, Bradbury & Blakey 1998, Birkhead *et al.* 1999, Martins 2003). However, it is not known whether a male's reproductive success is strongly affected by the attractiveness of its father, although this is the assumption on which Burley's studies were based. Rates of EPCs in the wild were found to be very low (Birkhead *et al.* 1990), but this is based on only a single study of one population. In fact, there is a fundamental lack of understanding of the relative fitness returns of producing male and female offspring. Hatching success of male and female eggs was not found to differ on either diet in Chapter 4, although male embryo mortality was greater for females mated to attractive males in Chapter 3, and higher male embryo mortality was also found by Rutkowska & Cichon (2002). There was no difference in post-hatching mortality between male and female chicks in either diet in Chapter 4, but both Bradbury & Blakey

(1998) and Kilner (1998) found that female mortality was greater than that of males on a poor diet.

The reason why females are more vulnerable to nutritional stress is not known. A hand rearing study has revealed that males gain mass faster than females on a restricted diet (Martins 2003), but it is important to also investigate the situation in the nest to look at whether males out-compete females, as has been found in blue tits (Oddie 2000), or whether there is some direct parental manipulation (Stamps *et al.* 1987, Yasukawa *et al.* 1990).

### **Maternal allocation of yolk androgens**

In Chapter 5, I investigated differences in yolk androgen deposition in relation to diet quality and found that, in the first round of the experiment (order effects suggested that females reacted more strongly to their experimental diet in this round), infertile HQ diet eggs contained greater concentrations of testosterone (T) than infertile LQ eggs. This suggested a greater investment, once again, in HQ diet eggs, which may be related to female condition (Pilz *et al.* 2003) or the expected rearing environment (Schwabl 1996b). However, in contrast to the egg mass data, there was no difference in androgen deposition in relation to laying sequence on the two diets. Both T and 5 $\alpha$ -dihydrotestosterone (DHT) decreased with position in the laying sequence, as has been previously reported in this species (Gil *et al.* 1999).

The lower concentration of T in HQ infertile eggs was thought to be because the embryos had begun to metabolise T (on the LQ diet there was no difference between fertile and infertile eggs, perhaps because initial deposition was lower and so embryonic development was slower). This hypothesis is supported by studies on Leghorn chickens, *Gallus domesticus*, which found that yolk levels of androstenedione, DHT and T decreased significantly during embryonic development, with significant declines in androgen levels evident as early as day one of incubation (Elf & Fivizzani 2002).

There was also evidence of differential investment in male and female eggs, again in line with predictions from sex allocation theory. On the LQ diet in round one, male eggs contained higher concentrations of T and DHT compared with female eggs and, for females that laid on the HQ diet in round one, female eggs tended to contain higher

concentrations of T and DHT than male eggs. This complex pattern of allocation may also reflect the balance of beneficial versus deleterious effects that have been found for T and DHT (Schwabl 1996a, Lipar & Ketteron 1999, Sockman & Schwabl 2000, Eising *et al.* 2001), and which may depend on the characteristics of the egg, including its size, position in the laying sequence and sex (Groothuis & Schwabl 2002). Research on yolk androgens and their effects in birds is still at a very early stage and there are clearly large gaps in our knowledge concerning the extent of control the laying female has over the allocation of androgens to her eggs including the detection or production of male and female eggs. There is also comparatively little known about the effects of androgens on the offspring, and the chick's ability to modify its situation through its own production and metabolism of T and DHT.

Chapter 6 examined differences in macro-composition of eggs laid on a HQ and a LQ diet, since diet and egg mass were found to strongly affect pre- and post-hatching mortality. Dry and wet yolk mass were found to increase isometrically with egg mass, such that larger eggs contained absolutely (but not disproportionately) larger amounts of these components. Wet albumen was found to increase disproportionately with increasing egg mass. In chickens, wet albumen is thought to be primarily responsible for large skeletal size (Finkler *et al.* 1998), so this suggests that within-clutch differences in egg mass may indeed serve to enhance or mitigate the existence of competitive hierarchies in zebra finches.

In summary, Chapters 4, 5 and 6 found that larger, higher quality and better provisioned eggs were produced on a HQ diet, which resulted in the large differences in offspring quality seen in Chapter 4. An interesting next line of investigation would be to assess the importance of the quality of the egg on subsequent male and female long-term fitness, as separate from the effect of the rearing environment.

### **Reproductive trade-offs**

Reproduction is subject to many trade-offs, which is a central theme in differential allocation and in this thesis. Reproductive trade-offs may take many forms, both within and between reproductive attempts. In common terns, *Sterna hirundo*, females experimentally manipulated to lay, incubate and rear an extra egg, showed reduced chick

provisioning resulting in decreased chick growth and survival (Heaney & Monaghan 1995). Rooks, *Corvus frugilegus*, that were given enlarged broods to rear, laid on average a week later the following year and reared fewer young compared with unmanipulated controls (Røskaft 1985). Trade-offs also operate in the laboratory with *ad libitum* food availability. When brood size was manipulated in zebra finches, initiation of the subsequent clutch was advanced after raising a small brood and delayed after raising a large brood, although clutch size was unaffected (Deerenberg *et al.* 1996). Furthermore, offspring quality is compromised in large broods of zebra finches, since chicks from large broods were lighter, and had shorter tarsi and wings, compared with individuals from smaller broods (de Kogel 1997).

In Chapter 3, there was no evidence of a trade-off in egg mass between reproductive events, since females invested in a similar manner in both breeding rounds. However, there is the possibility that a trade-off took place within the reproductive event, so that females that invested more in egg production reduced their investment at the chick provisioning stage (Nager *et al.* 2000b). In addition, trade-offs may not be evident on the short term, in *ad libitum* food conditions. In long-term aviary studies of zebra finches, Burley (1986a) found that increased reproductive effort in terms of parental care, led to greater mortality among these individuals.

In Chapter 4, females on the HQ diet laid heavier second clutches after rearing their first brood, suggesting little evidence of a trade-off for their large investment. However, in round two, on the LQ diet, offspring hatched and fledged at lower masses than did offspring reared by females on the LQ diet in round one, suggesting that the trade-off may have manifested itself at this later stage, probably due to the lower quality diet. Most females that were on the LQ diet in round one abandoned their clutches after laying. Those that did rear young invested a large proportion of their resources in terms of both time and energy (the young took longer to reach independence, and females lost substantial amounts of body mass). Furthermore, they did not lay second clutches within the timescale of the first breeding round. In short-lived species such as zebra finches, investment should be tilted towards maximising current reproductive effort (Linden & Møller 1989) and, because of the unpredictability of future reproductive opportunities in the wild, zebra finches may routinely subject themselves to more reproductive stress than

would a long-lived species that is selected to maximise survivorship (Mauck & Grubb 1995).

### **Carry over effects**

In the crossover design experiments (Chapters 3, 4 & 5) there was evidence of order effects, such that the treatment a female received in the first breeding round affected certain aspects of her response in the second breeding round. In Chapter 3, this was particularly evident in the case of egg mass, and was perhaps a reflection of a priming effect that has been reported for mice *Mus musculus* (Charalambous *et al.* 2003). Mice that were manipulated to produce smaller litter sizes, also produced smaller litters in their second breeding attempt. In zebra finches this may be adaptive in preparing females physiologically for re-mating quickly with the same male. In Chapter 5 carry-over effects were also evident and may have reflected nutritional and condition carry-over effects from the HQ diet given to females in the first breeding round, as well as other influences such as being paired with the same male in the second round of the experiment.

Several important findings came out of these experiments with respect to such order effects. Firstly, experimental design, notably time between experimental treatments and previous experience, is of importance. If there is a priming effect, then this is likely to be more pronounced over a shorter time period, perhaps emulating successive reproductive attempts within a breeding season.

Whether or not females rear young in the experiment is also important for two reasons. Firstly, rearing chicks is costly (Heaney & Monaghan 1995) and so a female's condition will be more greatly affected than if she only lays and incubates eggs. This may be the reason for the greater carry-over effect in Chapter 5 (in which no chicks were reared) where females on the HQ diet in round one continued to lay heavier eggs in round two on the LQ diet, compared with females on the LQ diet in round one. This may also have explained why a sex ratio bias was not detected in a paired analysis in Chapter 5. Some aspects of the egg mass and androgen data in Chapter 5 showed order effects and some did not. This may have reflected differences in plasticity of reproductive traits, or have been because females are reacting both to their own body condition and to expected rearing conditions.

The second reason why chick rearing may be an important consideration in experimental designs is that it can alter a female's perception of her mate. There is substantial evidence in laboratory zebra finches that rearing chicks together re-enforces the permanency of the pair bond (Immelman 1959, cited by Zann 1996, Clayton 1990). In many crossover experiments on differential allocation in zebra finches, females were not given the opportunity to rear young (Balzer & Williams 1998, Gil *et al.* 1999, Zann & Runciman 2003) and, until the impact of this is investigated, this cannot be assumed to be of minor importance.

### **Zebra finches as a study model**

Zebra finches provide a useful study model because they breed so prolifically in the laboratory and are amenable to experimental manipulations. Laboratory studies on resource allocation in zebra finches consequently far out-number those carried out in the field. Generations of inbreeding means that in the search for adaptive behaviours, the relevance to their wild conspecifics must be questioned, especially when the laboratory environment is so far removed from their natural habitat, and many basic life history traits (such as the degree of hatching asynchrony) are reportedly very different in the wild (Zann 1996). However, laboratory zebra finches are extremely useful in the investigation of the flexibility of reproductive strategies employed by females and as a model species for the study of differential allocation. Furthermore, since differential sex allocation has been demonstrated both between and within clutches, zebra finches may yet provide the answer to one of the biggest challenges in sex allocation theory, namely the elucidation of the mechanism by which females can manipulate their brood sex ratios.

In conclusion, this thesis has demonstrated that zebra finches show a great degree of flexibility, especially in relation to resource quality, which is consistent with a species that faces uncertain ecological conditions during the reproductive season (Hirshfield & Tinkle 1975). Behavioural aspects (feeding rates, incubation behaviour, etc) were not examined here but, not surprisingly, have also been found to be flexible in zebra finches (Burley 1986b, Gorman & Nager 2003). In species with bi-parental care, male resource allocation may be equally important and the male's interests may sometimes be in conflict with those of the female. Both paternal and maternal effects, but particularly the latter, are

of growing interest as their potential to enhance the inheritance of sexually-selected traits and affect the rate and direction of evolution is increasingly being recognised (Sheldon 2000, Qvarnström & Price 2001). The zebra finch remains a useful model species in the pursuit of such studies.

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