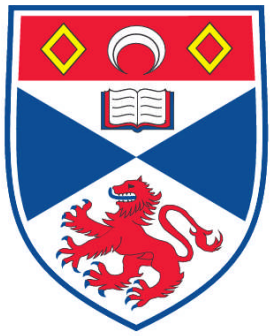
WILD AT HEART? : DIFFERENTIAL MATERNAL INVESTMENT IN WILD AND DOMESTICATED ZEBRA FINCHES (*TAENIOPYGIA GUTTATA*)

Emma C. Pariser

A Thesis Submitted for the Degree of PhD
at the
University of St. Andrews



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Wild at heart?

Differential maternal investment in wild and domesticated zebra finches (*Taeniopygia guttata*)

Emma Pariser

A thesis submitted to the University of St Andrews in application for the degree of Doctor of Philosophy

Supervisors:

Dr Jeff Graves

Dr Lucy Gilbert

Submitted: September 2009

I, Emma Pariser, hereby certify that this thesis, which is approximately 40,000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.
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Abstract

Over the past twenty years there has been an exponential increase in the investigation of maternal effects. Understanding the adaptive function of maternal allocation strategies is integral to interpreting the evolutionary outcomes of sexual selection. Thus, model animal systems that facilitate experimental manipulation and controlled investigation of the physiological and behavioural mechanisms underlying maternal effects are important to evolutionary biologists. The zebra finch (*Taeniopygia guttata*) has been used as a model to investigate avian life-history, signalling behaviour, neurophysiology, mate choice, and more recently, maternal effects. However, a potentially influential and rarely addressed problem with this species is the process of domestication. Within this thesis we aimed to both test current predominant maternal allocation hypotheses, but for the first time in both domesticated and wild zebra finches.

Chapter 2 develops on earlier work using domesticated zebra finches that has demonstrated differential allocation of maternally derived yolk androgens and antioxidants in eggs dependent on paternal attractiveness. This chapter specifically tests the ratio of these two yolk resources within individual eggs and shows that the balance of androgens to antioxidants varies by offspring sex and paternal attractiveness. Specifically, we found that mothers allocated a smaller androgen to antioxidant ratio to daughters when paired to green ringed (unattractive) males compared to red ringed (attractive) males. This pattern was reversed for sons, where mothers allocated a larger ratio of androgen to antioxidant when paired to red ringed (attractive) compared to green ringed (unattractive) males. We also show that brood sex ratio depended on both female condition and male attractiveness. It is concluded that investigating female allocation of individual resources within egg yolks may lead to incorrect assumptions on offspring fitness consequences, and that individual female state is an important consideration when predicting a resource allocation strategy.

Throughout this thesis colour bands are used as a method to manipulate male attractiveness. In chapter 3 the influence of these bands was further tested to elucidate whether they affect male behaviour or quality. Wild birds were used for this chapter as preferences for bands based on colour have only once been demonstrated in wild birds and it was felt this should also be replicated. We confirmed a female preference for males based on colour bands worn in mate choice trials, with red bands preferred

over green. Interestingly, we also found that colour of bands worn by males for an extended period in the single sex aviary influenced both their song rate and condition. Males that had worn red bands sang more in mate choice trials than both green banded or un-banded males. In addition red banded males were found to be in significantly better physical condition. These data suggest that earlier experiments in which it has been assumed that colour bands do not manipulate any form of intrinsic male quality should be re-evaluated.

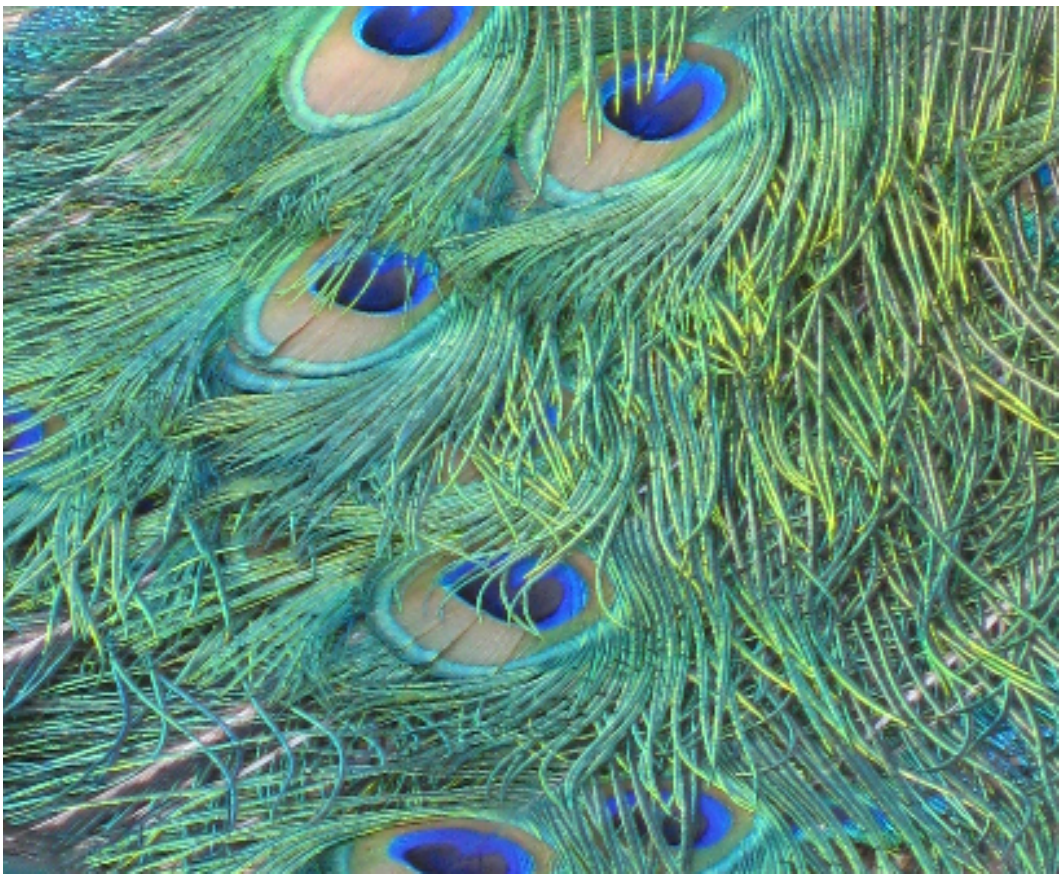
The final two data chapters, 4 and 5, return to investigating maternal allocation in response to male attractiveness, but for the first time in wild birds. Chapter 4 presents an experiment that was conducted on a wild, nest box breeding population of birds. Maternal resources allocation was investigated in both an experimental manipulation of male attractiveness, and also by correlating resource allocation with paternal phenotypic traits. A limited sample size meant few conclusions could be drawn from the experimental study, but significant positive correlations were found between both egg size and yolk testosterone (T) concentration and male phenotypic traits. This suggested that wild zebra finches may follow a positive investment strategy but requirs further investigation.

In chapter 5 experiments were repeated on wild birds that had been brought into captivity, to allow both an improved sample size and further control of influential environmental features. Again, female allocation strategies are tested using colour bands to manipulate male attractiveness, to allow direct comparisons with work on domesticated zebra finches. We found that females laid significantly heavier eggs for attractive compared to unattractive males, supporting the positive investment hypothesis. In addition we found an interaction between offspring size and paternal attractiveness treatment, with daughters of red banded (attractive) males being smaller than sons. This experiment is the first to demonstrate the influence of colour bands on maternal allocation in wild zebra finches and also provides further support for the positive investment hypothesis in this species. The final chapter discusses how overall patterns of female allocation were shown to be similar among wild and domesticated populations. It is concluded that demonstrated variations between populations and/or contexts reported in these studies cannot be explained by inherent differences between wild and domesticated individuals. Thus, the zebra finch remains a robust and reliable model for testing the evolution of avian maternal allocation strategies.

Chapter 1

Introducing sexual selection, mate choice, maternal effects & the zebra finch (*Taeniopygia guttata*)

Emma Pariser, Lucy Gilbert & Jeff Graves



Sexual selection & mate choice

"Sexual selection…depends, not on the struggle for existence, but on a struggle between males for the possession of the females; the result is not death to the unsuccessful competitor, but few or no offspring" (Darwin 1859, p.88)

Sexual selection is the mechanism used by Darwin to explain the evolution of conspicuous traits associated with a benefit in reproduction that did not obviously improve survival, and thus could not be explained by natural selection. He defined these traits as "sexual differences such as the greater size, strength and pugnacity of the male, his weapons of offence or means of defence against rivals, his gaudy colouring and various ornaments, his power of song and other such characters" (Darwin 1871). Darwin made a clear distinction between intersexual selection, in which mating success depends on an individual's ability to attract members of the opposite sex and intrasexual selection, in which members of one sex compete directly with one another for mating opportunities. The less controversial intrasexual selection has been widely accepted as the evolutionary process responsible for male characters such as large body size, and weapons such as horns and antlers, but intersexual selection (or mate choice) has been the subject of continued debate (Halliday 1983; Maynard Smith 1991; Kirkpatrick & Ryan 1991).

Mate choice can be defined as the tendency of one sex to mate non-randomly with respect to one or more varying traits in members of the opposite sex (Heisler et al 1987). This is easily explained if the choosy sex is gaining some form of direct benefit by selecting a preferred trait of the other sex; such as choosing males that are more fertile, offer greater parental care or provide superior resources (Heywood 1989). However, explaining the evolution of a preference when no direct benefits are received is more difficult and various models have been proposed. The first of these was proposed in 1930 by R.A. Fisher and is now known as the 'Fisherian run-away' theory, a model based on genetic coupling between the trait and preference. Alternatively, it has been proposed that preferences could evolve via a pre-existing bias or sensory trap for particular colours and/or sounds that can be exploited by males, a process referred to as sensory drive (Endler & Basolo 1998). Finally, another group of theories suggesting that secondary sexual signals are indicators of genetic quality (good-genes models) have been proposed by Fisher (1915), Williams (1966a)

and Zahavi (1975). These various mechanisms are believed to be important in preference evolution, especially if the trait is condition-dependent and develops in proportion to the physical quality of the individual (Andersson 1994). Current opinion among evolutionary theorists is abandoning a clear distinction between the various processes and viewing sensory drive as a likely explanation for providing an initial 'nudge' required to initiate choice-display co-evolution (Payne & Pagel 2000) and using some form of 'Fisher-Zahavi model' to describe the mechanism of preference evolution via indirect benefits (Eshel et al. 2000; Kokko et al. 2002).

Several empirical studies have provided support for various types of 'good genes' models (Anderssson 1994). However, these studies are often confounded by an inability to separate the father's genetic contribution from the genetic and material input of the mother, as well as the influence of environment during offspring development (Mousseau & Fox 1998). Laboratory experiments, by design, are capable of controlling for environmental effects; however other forms of parental effect are proving more complex and difficult to control. First, it is essential to understand what factors, other than the genetic quality of the parents, affect the future fitness of offspring. These can be broadly referred to as parental effects, although most studies have focused on the influence of the mother.

Maternal effects

Broadly speaking, maternal effects describe situations where a mother's phenotype influences her offspring's phenotype, or can be more formally defined as "the partial regression of offspring phenotype on mother's phenotype, holding genetic sources of variation constant" (Kirkpatrick & Lande 1989). Depending on the species, maternal phenotype can be the most important environmental condition experienced by an individual during development (Mousseau & Fox 1998). Mothers are able to make decisions about when and where to produce offspring, and in some species, the size, quality and sex of offspring (Trivers & Willard 1973). The understanding that these effects can have a major influence on an individual's fitness, and can respond to selection, has led to them becoming of increasing interest to evolutionary biologists. It has long been understood that mothers make a genetic contribution to their offspring - in most species half of the genetic effect on offspring phenotype (i.e. Mendelian inheritance). However, environmental influences attributed to the mother, for example

food provision and protection from predators, can also be considered as genetic when between-female differences reflect additive genetic variation underlying these behaviours (Moore et al. 1998). This has been described as a form of indirect genetic effect; a term used to encompass the influence of all relatives upon an offspring's phenotype, which can be used to contrast with direct genetic effects (Wolf et al. 1998). A further consideration is the influence of environment translated via mothers to their young, which has been termed 'indirect environmental effects' (Groothius et al. 2005b). For example in all altricial species, as environmental conditions such as food availability or quality fluctuate, consequential nutritional effects are translated to the offspring via the mother as the provider of their food. These various effects all contribute to offspring development (figure 1).

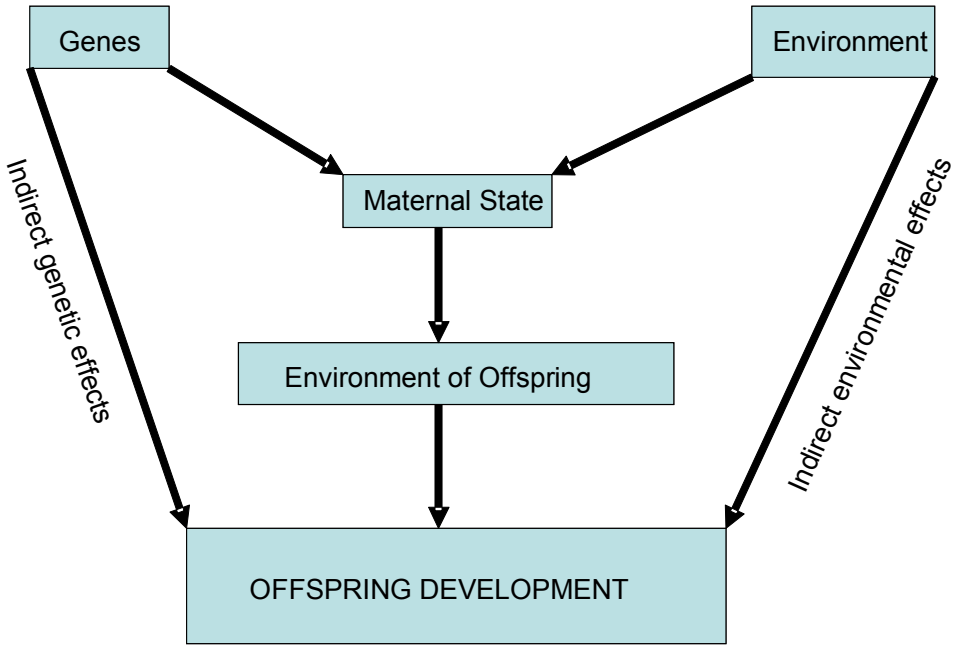


Figure 1: Various maternal effects acting on offspring development (adapted from Groothius et al., 2005b).

It is generally accepted and understood that each of these factors are important in determining the outcome of development. However, the more complex questions lie in whether mothers are capable of controlling some or all of these effects, and whether they can be adaptive, rather than simply physiological side effects (Heath &

Blouw 1998). A recent and growing body of research has begun to test these questions and has shown that maternal effects can indeed function as adaptive mechanisms allowing mothers to affect offspring development in fluctuating environments (Mousseau & Fox 1998; Mousseau et al. 2009).

Life-history theory

Life-history theory predicts that females should alter their investment in a particular breeding attempt according to the likelihood of its success (Williams 1966b). A female should therefore use all available cues in her environment to make appropriate allocation decisions. One aspect of the environment that can be an important predictor of reproductive success is mate quality. Females use male phenotype as a cue to provide information on the genetic and/or direct effects likely to be conferred in a mating event.

Differential allocation theory:

There are currently two main theories predicting how females will differentially allocate resources in response to variations in mate attractiveness:

1. Positive Investment

This theory was originally proposed by Nancy Burley (1986a) and was termed the differential allocation hypothesis. Burley proposed that females of iteroparous species should increase allocation of resources to offspring sired by attractive males compared to a reduced investment for poor quality, unattractive males. This is based on the logic that mothers trade off between current and future reproductive events, and that they only have a limited budget of resources to allocate (Sheldon 2000). Predictions from this theory have been empirically demonstrated in a wide range of taxa (reviewed in Sheldon 2000; and more recent studies include Bretman et al. 2006; Head et al. 2006; Skinner & Watt 2007; Dentressangle et al. 2008; Helfenstein et al. 2008; LaDage et al. 2008; Kingma et al. 2009; Silva et al. 2009). 'Positive investment' refers to the positive relationship between the maternal allocation of resources and male sexually selected traits and does not infer beneficial investment.

2. Compensatory Investment

More recently it has been suggested that females should increase investment when paired to unattractive males, compared to attractive males, in order to compensate for

their reduced quality and mitigate any negative effects on offspring fitness (Gowaty et al. 2003; Bluhm & Gowaty 2004; Gowaty et al. 2007; Gowaty 2008). This idea has found support among some studies (Saino et al. 2002; Michl et al. 2004; Navara et al. 2006; Bolund et al. 2009), although so far less than for the positive investment hypothesis. This theory also predicts a relationship between female investment and male sexually selected traits, although in this case the slope of the relationship is in the opposite direction, as depicted in figure 2.

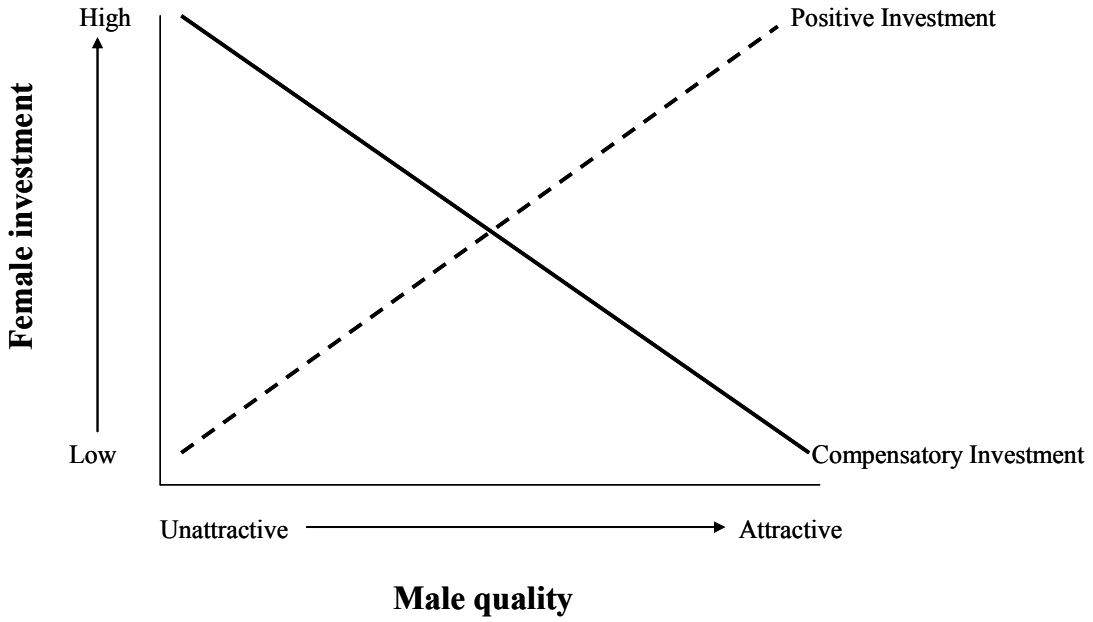


Figure 2: Diagram depicting the direction of the relationship between female investment and male attractiveness for two main theories, positive investment (dotted line) and compensatory investment (solid line).

Interestingly, there is current support for both hypotheses from experiments conducted using the same species. Mallard (*Anas platyrhynchos*) females have been shown to lay larger eggs for attractive males compared to unattractive males in one study (Cunningham & Russell 2000), while the opposite allocation pattern has also been demonstrated (Bluhm & Gowaty 2004). It is important to note that for all studies the resources allocated are always compared between attractive and unattractive males. Therefore it is just as likely that the patterns seen are driven by a reduction in

investment for one treatment group rather than an increase for the other. Female zebra finches (*Taeniopygia guttata*) have been found to have higher deposition of yolk androgens and antioxidants, as well as egg size, for attractive males (Gil et al. 1999; Rutstein et al. 2004a; Gilbert et al. 2006; Williamson et al. 2006), but a more recent study found that females paired to unattractive males laid larger eggs (Bolund et al. 2009). It seems, therefore, that discrepancies between female allocation strategies cannot be explained by species-specific differences. A recent model using the state-based approach, in which an individual's state (such as age or physical condition) is also incorporated within the model parameters, has tested the circumstances under which each hypothesis is likely to be upheld (Harris & Uller 2009). In accord with studies published to date, the authors found that positive investment was predicted across a range of biological parameters and thus likely to be seen among species with varying life-history strategies (Harris & Uller 2009). Conversely, their model only predicts compensatory investment under a limited set of conditions. Specifically, the energetic state of breeding females and timing of reproduction strongly influenced whether positive or compensatory investment were selected as investment strategies (Harris & Uller 2009), which may explain the within species discrepancies seen to date.

To accurately test predictions produced by these theories, experimental manipulation to isolate and disseminate confounding influences (e.g. direct genetic benefits gained from attractive males, attractive males selecting superior females, attractive males controlling better territories or higher food availability) is essential. This requires a technique that combines manipulation (altering phenotype) or objective scoring of male attractiveness with random assignment of mates. Any data obtained in this way also contributes to the discussion in current literature regarding the influence of maternal effects upon 'good genes' models of sexual selection. It has been suggested that finding differential investment by the female can confound the results that have been previously explained by a male's genetic quality alone (Gil et al. 1999; Cunningham & Russell 2000; Møller & Thornhill 1998). It has been argued that a female that allocates more resources to the offspring of an attractive male actually promotes sexual selection by amplifying the selection pressure on the trait and hence its representation or value in subsequent generations (Sheldon 2000; Wolf et al. 1998). Kotiaho et al (2003) provided support for this idea, finding a significant heritability of horn length and body size, but when differential maternal allocation

was controlled for the observed estimates of genetic variance were greatly reduced. This suggests that differential female investment was helping to maintain high coefficients of variation observed for fitness-related traits in this species. If this phenomenon is found to be common across multiple taxa, differential investment will play an important role in sexual selection, speciation, and evolutionary biology in general (Sheldon 2000; Wolf et al. 1998; Higashi et al. 1999).

Sex allocation:

In addition to adaptively manipulating resources in response to a fluctuating environment, some theories predict that females will adjust their allocation to offspring dependent on their sex. Charnov (1982) predicted, in his classic text, that under certain conditions offspring sex ratios could deviate from parity. This is expected to occur in circumstances where the relative fitness benefits accrued from producing sons or daughters differs, for example in environments where one sex has a higher variance in reproductive success than the other (Trivers & Willard 1973). If a female mates with a poor quality, unattractive male, it would be beneficial to bias the offspring sex ratio in favour of the least variable sex (usually female). Alternatively, another theory predicts that if sons inherit their father's attractive secondary sexual traits it would be sensible for females to produce more sons for attractive fathers; known as the 'sexy-son hypothesis' (Weatherhead & Robertson 1979). *Prima facie* these theories predict sex-ratio adjustment in the same direction, however, this varies between species and conditions. The mechanisms of sex-ratio adjustment can be either primary (i.e. upon fertilization) or secondary, where resources are directed towards the favoured sex, thus selectively reducing the chances of survival for the other sex (Charnov 1982; Stamps 1990).

The zebra finch (*Taeniopygia guttata*)

The zebra finch is a sexually dimorphic, Australian passerine that is easily kept in captivity. Although a colonially breeding bird, they are socially monogamous with very low levels of extra-pair copulation (Birkhead et al. 1990), and are thought to pair for life (average life expectancy in the wild is one year, Zann 1996). Both sexes exert mate choice, with female choice believed to be the dominant force driving pair formation (Burley 1986b). They have an extended breeding season and have been

described as opportunistic breeders that will readily breed in captivity if given the correct conditions (Zann 1996). Males possess numerous sexually selected traits which have been comprehensively investigated, with over 30 publications investigating zebra finch mate choice (reviewed in Zann 1996; Forstmeier & Birkhead 2004). In addition, it has been found that mate attractiveness can be manipulated using specific artificial ornaments (Burley 1981; Burley et al. 1982, Burley 1985, 1986a; Hunt et al. 1997). Both sexes show mate preferences dependent on the colour of leg bands worn by conspecifics. Females find red bands attractive, green bands unattractive, while orange bands do not influence mate choice decisions. Males find females wearing black colour bands attractive, blue bands unattractive and again orange is neutral with respect to mate preference (Burley et al. 1986; Burley 1988b). It is not understood why these colours influence preferences in both males and females.

The ability to either score male attractiveness, or artificially manipulate phenotype quality by simply adding colour bands, makes this species ideal for investigating maternal differential allocation strategies. For this reason, the zebra finch was used as a model for the work presented in this thesis, as well as much of the published data comprising the avian maternal resource allocation literature.

Avian maternal resource allocation (focusing on the zebra finch)

Birds are probably the most thoroughly researched taxon in the field of behavioural ecology, so it is of no surprise that they feature heavily in studies investigating differential allocation and adaptive maternal effects. This is partly due to the taxon as a whole offering a well documented array of reproductive strategies and mate choice behaviours. It is also because avian reproduction is characterised by a series of well defined steps; egg laying, incubation, brooding, and provisioning offspring; each of which is easy to separate and study. Importantly, female birds provide all the resources needed for embryonic growth and development at one point in time, in an external egg which can be easily measured and manipulated. Variation in resource allocation at each of these stages has been shown to have important consequences for offspring development and/or fitness (Price 1998).

Offspring Sex Ratio

Pre-ovulatory mechanisms of sex-ratio adjustment, although they remain unknown, have been suggested as the most efficient means of adaptive sex allocation (Komdeur et al. 2002). A growing number of studies have found that birds are capable of varying clutch sex-ratio at laying. The majority of these studies have found that sex-ratio is skewed in response to variation in maternal condition or food availability (Lessells et al. 1996; Appleby et al. 1997; Heinsohn et al. 1997; Komdeur et al. 1997, 2002; Bradbury & Blakey 1998; Nishiumi 1998; Nager et al. 1999; Pike 2005; Whittingham et al. 2005). However, some investigators have shown that mate attractiveness may also influence the pattern of sex-ratios within clutches. Ellegren et al. (1996) found that female collared flycatchers (*Ficedula albicollis*) vary the primary sex-ratio of clutches dependent on the attractiveness of their mate. This has also been found in great tits, *Parus major,* (Kolliker et al. 1999) and blue tits, *Cyanistes caeruleus* (Svennson & Nilsson 1996; Sheldon et al. 1999) although this is not always consistent between years (Griffith et al. 2003). Conversely, there is a growing list of studies on various avian species that have failed to find such an effect, including those using zebra finches (Saino et al. 1999; Westerdahl et al. 1997; Leech et al. 2001; Radford & Blakey 2000; Zann & Runcimann 2003; von Engelhardt 2004; Rutstein et al. 2004b). Whether the dynamics of differential sex allocation with respect to mate attractiveness are environment- or species-specific, or more influenced by variation in experimental design, is unclear at this stage.

Clutch size

In the zebra finch, clutch size has been found to increase when females are given social stimulus in the form of sounds from their colony (Waas et al. 2005), which is thought to play a part in maintaining colonial breeding in this species. The seasonal timing of breeding is also influential in both wild (Frith & Tilt 1959; Zann, 1994) and domesticated birds (Williamson et al. 2008), with larger clutches being laid in the summer months. Diet is also an important factor, with a high quality diet increasing the clutch size a female is able to lay (Rutstein et al. 2004c). Also, the diet received in the first month of life has been shown to permanently influence the clutch size that a female lays (Heywood & Perrins 1992). However, only one study has found a "positive but weak" effect of mate attractiveness on clutch size (Balzer & Williams 1998). This experiment scored males as 'preferred' or 'non-preferred' via mate choice

trials. Other studies that have artificially manipulated male attractiveness using colour bands have found no effect of male quality on clutch size in this species (Burley 1986a; von Engelhardt 2004; Gorman et al. 2005 Williamson et al. 2005; Bolund et al. 2009).

Egg size

Egg size in birds has often been found to correlate with offspring mass and skeletal size during the early post-hatching period (Christians 2002). This is a crucial stage in development and a period of high offspring mortality. Fluctuating egg size in relation to mate quality has been demonstrated in several species: Female mallard ducks (*Anas platyrhynchos*) produce larger (volume) eggs when mated to attractive males (Cunningham & Russell 2000). Larger (mass) eggs have also been found to be produced by female Chinese quail (*Coturnix chinesis*) when paired with attractive males expressing large sexual ornaments (Uller et al. 2005) and by female ostriches (*Struthio camelus*) in response to male colouration (Bonato et al. 2009). In zebra finches, a direct trade-off between clutch size and egg size has previously been shown (Williams 2001). Early experiments on wild birds found egg size to be consistent within individual females (Zann 1996), but this can also be influenced by seasonality in both wild and captive populations (Williamson et al. 2008). High between-individual variation in egg size is believed to be common in bird species (reviewed in Christians 2002). In zebra finches, however, experiments that have accounted for this variability have demonstrated repeatable treatment effects on egg size. When fed a high quality diet, females will lay heavier eggs, increasing weight with laying order. Conversely, on a low quality diet the eggs are lighter and decrease in weight with laying order (Rutstein et al. 2004c). With respect to mate attractiveness, Williamson (2005) found greater incremental egg weight when laid for red-banded males, while Bolund et al. (2009) found the opposite; increased egg size for unattractive males. Variation in egg size across the laying order has been suggested as a mechanism for females to control the effects of hatching asynchrony (Slagsvold et al. 1984). Hatching asynchrony is common among birds and is due to females starting to incubate prior to clutch completion. This causes some offspring to hatch earlier than their siblings, establishing a brood hierarchy, which has been proposed as a means of adaptive brood reduction in stochastic environments (Clark & Wilson 1981). However, increasing egg size in later laid eggs is believed to help offset the costs of

delayed hatching. This idea has been extended by Schwabl (1993) to incorporate variation in egg contents.

Egg content

Eggs contain all the nutrients, proteins, fat, minerals, vitamins and water required for successful offspring development (Romanoff 1960). However, it was not until relatively recently that maternally derived androgens were discovered in avian yolk (Schwabl 1993). Androgens are thought to have strong effects on offspring phenotype, so the idea that mothers could vary their deposition among eggs (Schwabl 1993) was of immediate interest to behavioural ecologists (Groothuis et al. 2005a). In addition to androgens, egg yolks contain relatively large volumes of antioxidants, also maternally derived and essential for offspring growth and development (Surai 2002). Offspring fitness may be influenced not only by the independent qualities of any of these resources, but also their concentrations relative to each other (Royle et al. 2001).

Yolk androgens

The mechanisms by which females can adaptively allocate hormones to their egg yolks are currently unknown, although three main theories are currently being investigated (reviewed by Groothuis & Schwabl 2008). Despite this, there is evidence that females can adaptively vary yolk androgen levels in response to their environment (Gil 2003; Groothuis et al. 2005b). Specifically, females have consistently been shown to differentially allocate yolk androgens in response to male attractiveness. Domesticated female zebra finches deposit more testosterone (T) and 5α-dihydrotestosterone (DHT) into eggs sired by attractive (red banded) males (Gil at al. 1999; von Engelhardt 2004). Canaries (*Serinus canaria*) deposit higher concentrations of yolk testosterone when exposed to an attractive song consisting of a complex repertoire of 'sexy' syllables (Gil et al. 2004; Tanvez et al. 2004 but see Marshall et al. 2005). Female barn-swallows (*Hirundo rustica*) and peafowl (*Pavo cristatus*) increase their deposition of yolk testosterone when paired to males with artificially enhanced tails (Gil et al. 2006b; Loyau et al. 2007). Further, these types of adjustments by females can be surprisingly rapid, with female blue tits (*Cyanistes caeruleus*) altering allocation within a clutch following manipulations of male attractiveness after the second laid egg (Kingma et al. 2009). However, although these experiments demonstrate the existence of maternal effects, they cannot be interpreted

as support for the positive investment hypothesis unless yolk androgens are found to be beneficial to offspring and costly for mothers to produce.

The consequences of increased yolk androgen levels for developing birds has been studied by injecting eggs with testosterone (reviewed in Groothius et al. 2005b). Direct physiological effects include a higher probability of offspring hatching (Schwabl 1993) and shorter incubation periods (Eising & Groothius 2003). Offspring growth rate has been found to be increased in many species (Schwabl 1996a; Eising & Groothius 2003; Navara et al. 2005; Tschirren et al. 2005; Muller et al. 2009). However, in zebra finches increased yolk T only increased growth in female offspring (von Engelhardt et al. 2006; but see also Tobler et al. 2007). Sex-differences in the influence of yolk T have repeatedly been found (Muller et al. 2002; Rutstein et al. 2004b; Gilbert et al. 2005; Rutkowska & Badyaev 2008; Pitala et al. 2009), suggesting an involvement in maternal control of secondary or primary sex-ratio adjustment. Increased early growth is not however necessarily beneficial as it may incur costs later in life for many birds (Metcalf & Monaghan 2001; Tschirren et al. 2009).

The effects of increased testosterone have also been shown to have clear negative influences on offspring. Responses to artificially increased yolk T have included delayed hatching (Sockman & Schwabl 2000; von Engelhardt et al. 2006) and increased mortality (Sockman & Schwabl 2000; Navara et al. 2005; Muller et al. 2009). Additional to these costs, there has been empirical support for the existence of a direct trade-off between yolk androgen concentration and offspring immune function (Groothius et al. 2005a; Navara et al. 2005; Cucco et al. 2008; Sandell et al. 2009; Tobler & Sandell 2009). The possibility of such a trade-off has led to increased interest in yolk antioxidants and their interaction with androgens, as antioxidants are known to play a role in avian immune function and are maternally deposited into egg yolks (Surai 2002).

Yolk antioxidants

All organisms require antioxidants to neutralise free radicals and oxidants that are produced as side-effects of normal metabolic activity (Surai 2002). Carotenoids and vitamin E are both antioxidants that can only be obtained from the diet, possibly in limited quantities, and are both found in avian egg yolk. In fact, it is the carotenoids that give yolk its colour (Blount et al. 2000). Vitamin E is the name given to a group

of molecules; α-, β-, γ-, δ-tocopherol/ tocotrienol (Surai 2002). These molecules are produced by higher plants; tocopherols are produced in the green, leafy tissues and tocotrienols are found in the germ and seeds (Halliwell & Gutteridge 1999). Carotenoids are synthesized by photosynthetic bacteria, fungi and plants, and are known to have multiple uses in birds (Surai 2002). They are not only involved in protection against oxidative damage, but are believed to be important in immune function (Olson & Owens 1998), and are precursors of vitamin A, essential for development in vertebrates (Zile 2002). Carotenoids also act synergistically with Vitamin E by slowing its depletion from binding excess free radicals (Bohm et al. 1997).

Birds are among the many vertebrate taxa that utilise carotenoid pigments in both secondary sexual signals and immune defence. The trade-off between these two functions has been suggested as a mechanism for producing honest signals of condition (von Schantz 1999). Empirically support for this has been found in zebra finches, where manipulation of dietary carotenoids levels explained parallel variation in immune function and attractiveness (beak colour) in males (Blount et al. 2003b). A related trade-off is thought to exist where mothers allocate antioxidants to their offspring via egg yolk (Blount et al. 2000). Antioxidants are an important limited resource for mothers, but are also crucial to offspring for protection against lipid peroxidation during growth and development (Surai & Speake 1998; McGraw et al. 2005; Biard et al. 2006; Berthouly et al. 2008; De Neve et al. 2008). Studies investigating natural variation in yolk antioxidants have found that in most species concentration decreases with laying order (Royle et al. 1999, 2003; Saino et al. 2002; Williamson et al 2006; but see also Newbrey et al. 2008). This pattern is thought to be physiologically constrained, since it does not vary when birds receive supplementary food (Blount et al. 2002; Royle et al. 2003). However, adaptive allocation of yolk carotenoids in response to male attractiveness has been demonstrated in two species. In the zebra finch contradictory results have been reported, with females increasing allocation of yolk carotenoids in later laid eggs when paired to attractive males in one study (Williamson et al. 2006), supporting the positive investment hypothesis, while in a second study higher levels of carotenoids (quantified via yolk colour) were deposited in eggs laid for unattractive males (Bolund et al. 2009), reflecting compensatory investment. Additional support for the compensatory investment hypothesis was reported by Saino et al. (2002), who found that female barn swallows

increased yolk carotenoid deposition when paired to males with experimentally reduced attractiveness (shortened tails).

Yolk androgen : antioxidant ratio

A final consideration regarding maternal effects and egg quality is the interactions between various yolk constituents. Little is known about the balance of yolk androgen and antioxidant concentrations or whether they interact, as studies to date have predominately focused on either androgens or antioxidants independently. It is thought that yolk androgens and antioxidants may have complementary but opposing effects (Royle et al. 2001).

In two studies on black-backed gulls, females were found to increase androgen deposition with laying order, while simultaneously decreasing antioxidant concentrations (Royle et al. 2001; Groothuis et al. 2006). Royle et al. suggest that this may be an adaptive mechanism to either aid or abate brood reduction in response to varying food availability. Androgens are thought to have negative effects on the immune system (Ketterson & Nolan 1999), impair enzyme antioxidants and induce oxidative stress (von Shantz et al. 1999). Androgens are therefore likely to have direct effects on the quantity and action of yolk antioxidants available to the developing embryo. Consequently, females could reduce the chances of offspring survival in later laid eggs by increasing androgen content relative to antioxidants. Royle et al. (2001) suggest that this effect would vary depending on environmental conditions such as food availability or possibly mate attractiveness. Two studies have measured both androgens and antioxidants in egg yolks in relation to social stimuli (Verboven et al. 2005; Hargitai et al. 2009) but neither presented data on the ratio within individual eggs. Similarly, correlations between yolk androgen and antioxidant levels and paternal attractiveness/quality were made in the barn swallow (Safran et al. 2008), but relative proportions were not reported. These omitted data are necessary to investigate whether birds can adaptively vary relative proportions of these substances in eggs, as suggested by Royle et al (2001).

The domesticated zebra finch

Model species have been extensively used in biology, to increase understanding of a range of topics from physiology, genetics, endocrinology, animal behaviour and evolutionary ecology. Generally, species that have been developed as a model system for their taxon, such as mice, guppies, *Drosophila* or zebra finches, have become so due to their ability to survive and reproduce quickly under laboratory conditions. It could be argued, however, that for the investigation of evolutionary questions, the use of such animals may not be appropriate. Animals living and breeding in captivity are exposed to a suite of different stressors and selective pressures compared to their wild counterparts. Over multiple generations, this has the potential to produce an animal that bears little resemblance, both behaviourally and physically, to its wild ancestor (Darwin 1859).

The zebra finch may be particularly vulnerable to this process due to two additional factors: First, in 1960 the Australian government banned the export of all native wildlife, meaning that birds sourced for use in experiments comprising the majority of published work on this species (from European and North American institutions) have been bred in captivity for over 40 years. As these birds can reach sexual maturity at 100 days (Zann 1996), this equates to a large number of generations with little or no genetic input from wild stock. Second, zebra finches have been a popular pet bird worldwide and have been selectively line bred by aviculturalists to produce an array of different colour morphs that do not exist in the wild. This has resulted in a domesticated bird that has significantly increased in body size and assortatively pairs when given a choice between wild or domesticated partners (Rutstein et al. 2007). Reduced genetic variability in domesticated birds compared to their wild ancestors has also been demonstrated, along with significant differentiation between captive study populations around the world (Forstmeier et al. 2007). It has been suggested that this variability may partially explain discrepancies in studies between research groups. Very little work has been performed using wild zebra finches, especially with a focus on maternal allocation strategies (Zann 1996). Such data would provide valuable information on male and female sexual behaviour and response to standard manipulations, in the same ecological context that these behaviours have evolved.

Aims

The primary aim of this study was to investigate differential allocation of maternal resources at the egg laying stage, in response to mate attractiveness. Male attractiveness was experimentally manipulated by the addition of coloured leg bands and within-female variation in investment was quantified for a range of maternally derived resources. Similar questions were tested in domesticated, free-living wild and captive wild-caught zebra finch populations to additionally investigate variation that could be attributed to domestication in this species.

The first experiment (chapter 2) was conducted using domesticated birds housed in the animal facility at St Andrews University. This study was focused on extending earlier work that had found positive investment of androgens (Gil et al. 1999) and antioxidants (Williamson et al. 2006) in response to male attractiveness in zebra finches. Specifically, we were interested in investigating whether the ratio of these two resources was allocated to offspring in a predictably adaptive manner; with respect to mate attractiveness and offspring sex ratio hypotheses.

To date, only one study has investigated the influence of coloured leg bands on wild birds (Burley 1988), thus it was important to replicate and confirm these results (chapter 3). This study also sought to further investigate any potential influence of colour bands on mediating intrasexual interactions (Cuthill et al. 1997). This study contributed to a greater understanding of the impact of artificial ornaments on socially breeding birds and allowed for a more accurate interpretation of other studies, including those from this thesis.

Finally, we conducted repeated experiments to investigate within female allocation of resources in wild zebra finches. To achieve this, we first conducted an experiment using a free-living breeding population (chapter 4). This gave useful insight into natural variation in maternally derived resources, such as yolk testosterone concentrations, which is commonly measured in domesticated birds but never measured before in the wild. Following this, an identical experiment was conducted in captivity, using wild-caught birds (chapter 5) to more rigorously investigate maternal allocation by controlling environmental variation. This was conducted in large outdoor aviaries to encourage natural breeding behaviour and allow for investigation of extra-pair courtship. By assigning true genetic paternity to offspring, data were not confounded by extra-pair parentage.

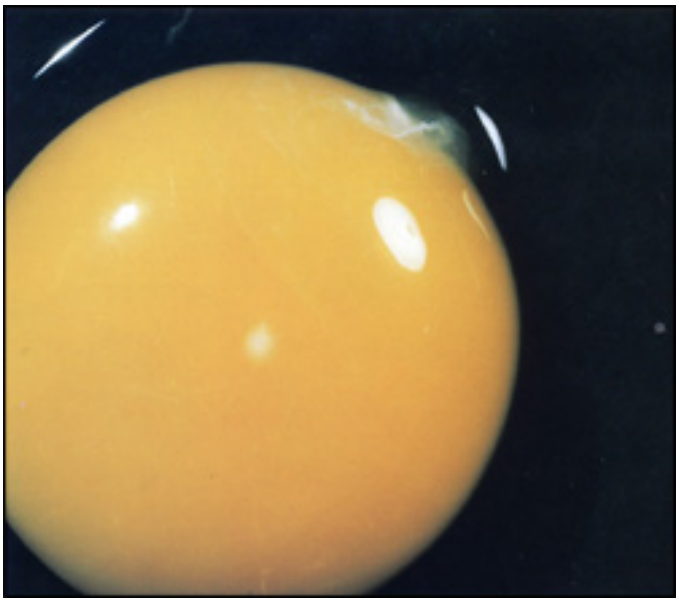
The findings of each experiment presented in this thesis contribute, in some way, to the ongoing debate over adaptive explanations for maternal allocation strategies. Results are discussed and interpreted in relation to the contrasting predictions of the positive investment (Burley 1986; Sheldon 2000) and compensatory investment (Gowaty 2008) hypotheses. The concluding chapter will synthesise the overall findings, and determine how they contribute to the available evidence for these competing theories. Additionally, by using wild birds, these studies have incorporated a novel dimension to the existing literature.

All experiments other than chapter 2 were conducted using wild birds, both in the field (chapter 4) and in captivity (chapter 5) in Australia. To aid comparisons between these and previous studies, standard methodologies for manipulating male attractiveness and quantifying female investment were employed.

Chapter 2

Mind the Gap: the ratio of yolk androgens and antioxidants varies between sons and daughters dependent on paternal attractiveness.

Emma Pariser, Lucy Gilbert, Kate Arnold, Neil Hazon, & Jeff Graves



I collected 100% of data and carried out 100 % of analysis and write-up, with comments and advice from LG and JAG.

Abstract

Females are expected to partition resources between offspring in a context-dependent way so as to maximise total fitness returns from a reproductive attempt. Defining a beneficial resource, however, is not always straightforward. It is known that female zebra finches (*Taeniopygia guttata)* can adaptively vary the allocation of yolk androgens and antioxidants among offspring. Yet it may be the balance of allocation between these yolk constituents that has more important fitness consequences for developing young than the independent effects of each. We tested whether the relative allocation of these two resources can vary predictably according to either positive or compensatory maternal investment hypotheses. We manipulated male attractiveness using coloured leg rings, measured individual female condition and randomly paired birds for breeding. Female allocation of yolk androgens and antioxidants to each egg was quantified and all offspring were sexed. We then analysed the ratio of the two constituents within and between clutches and between offspring sexes, in relation to female condition, male attractiveness and clutch sex ratio. We found that females allocated a smaller androgen to antioxidant ratio to daughters when paired to green ringed (unattractive) males compared to red ringed (attractive) males. This pattern was reversed for sons, where mothers allocated a larger ratio of androgen to antioxidant when paired to red ringed compared to green ringed males. We also found that brood sex ratio depended on both female condition and male attractiveness. Our findings show that female zebra finches vary the balance of resources within and between clutches in relation to mate attractiveness.

Introduction

Life-history theory predicts that mothers should alter their investment in a particular breeding attempt according to the perceived value of that event (Williams 1966; Sheldon 2000). This value can be potentially assessed by females using environmental cues such as food availability, body condition (Rutstein et al. 2004b; Rutstein et al. 2004c) and the attractiveness/quality of mates. Assuming that females trade off

investment between their current and future reproductive events, the positive investment hypothesis predicts that female investment will be higher when paired with an attractive mate, and lower when paired with an unattractive mate. Support for this theory has been found across a variety of avian species (Burley 1988a; Petrie & Williams 1993; Cunningham & Russell 2000). The hypothesis is generally applied to species where multiple breeding attempts with different mates are common (reviewed by Sheldon 2000). Conversely, the compensatory investment hypothesis predicts that females will allocate more resources when paired with unattractive males as a means of compensating for (a) the predicted reduction in paternal care due to the male's inferior quality and/or (b) the predicted inferior genetic quality of any offspring. This theory has received less attention in the published literature, but it has found empirical support in some studies (Saino et al. 2002; Navara et al. 2006; Bolund et al. 2009).

In addition to between-brood variations in resource allocation, it has been predicted that mothers may allocate resources differently between individual offspring within a brood. For instance, the Trivers-Willard hypothesis states that mothers will, depending on their own condition, alter offspring sex ratio or investment between the sexes in favour of offspring that have the highest reproductive value (Trivers & Willard 1973). In species where males have a higher condition-dependent variance in reproductive success than do females, this hypothesis predicts that mothers in good condition will invest more in sons or produce a male-biased sex ratio, while mothers in poor condition will invest more in daughters or produce a female-biased sex ratio (Trivers & Willard 1973). If birds are capable of primary sex-ratio adjustment this variation could be observed at laying. Alternatively, secondary sex-ratio adjustment could result from variation in the allocation of resources to offspring within a brood, dependent on their sex.

Bird eggs at laying contain all the essential nutrients and resources necessary for the offspring's development: essential proteins, fat, minerals, vitamins and water (Romanoff 1960), all of which are maternally derived and potentially costly to provide (Monaghan & Nager 1997). Egg yolk also contains maternally derived hormones and antioxidants, which both play an important role in embryo development and potentially influence the eventual phenotype of offspring (Surai 2002; Groothuis et al. 2005a).

It has been shown that both hormone and antioxidant levels may vary in egg yolks in relation to environmental factors, such as mate attractiveness (Gil et al. 1999;

Gil et al. 2004; Rutstein et al. 2004a; von Engelhardt 2004; Rutstein et al. 2005; Williamson et al. 2006). The effects of these yolk constituents, specifically androgens, on developing birds has been studied by injecting eggs with testosterone (reviewed in Groothius et al. 2005b). The potential benefits of increased yolk androgen on offspring include; increased hatching success, shorter incubation periods, increased growth and decreased mortality (Schwabl 1993, 1996; Eising & Groothius 2003; Pilz et al. 2004; Navara et al. 2005; Tschirren et al. 2005; von Engelhardt et al. 2006). However, higher concentrations of androgen have also been shown to produce negative effects in offspring such as increased mortality (Sockman & Schwabl 2000; Navara et al. 2005). A known cost associated with higher androgen concentrations is the direct trade-off with immune response, specifically T-cell immunity (Dufty et al. 2002; Groothius et al. 2005a; Navara et al. 2005, Cucco et al. 2008; Sandell et al. 2009). High androgen concentrations may also impair antioxidant defences and induce oxidative stress (von Schantz et al. 1999). Thus, a potential benefit of maternally derived antioxidants in egg yolk could be to help mitigate the costs associated with maternally-derived androgens (Royle et al. 2001). Further support for the direct interaction of prenatal androgens on antioxidant capacity was found in a recent study showing reduced plasma antioxidants in zebra finch chicks hatched from eggs injected with additional testosterone (Tobler & Sandell 2009). Antioxidants such as carotenoids and vitamin E are known to be critical for embryonic development, as they protect growing tissues from oxidative damage (Surai 2002). Supplementary feeding studies on zebra finches have also demonstrated how antioxidants can enhance immune function in adults (Blount et al 2003; McGraw & Ardia 2003). A similar effect was shown in early post hatching barn swallows following a direct manipulation of the yolk concentration of antioxidants (Saino et al. 2003). Deposition of antioxidants into eggs also represents a trade-off for mothers, since antioxidants are a limited resource that can be obtained only from the diet, and must be shared between her own requirements and those of her developing offspring (Blount et al. 2004).

Little is known about the balance of these two potentially interacting resources within the egg yolk, since work has predominately focused on either androgens or antioxidants independently. The few studies that have investigated the inter-relationships of yolk androgens and antioxidants have presented correlational data from wild populations of birds (Royle et al. 2001; Groothuis et al. 2006; Navara et al.

2006; Safran et al. 2008). Ideally, in order to test for subtle changes in egg composition in response to environmental conditions it is important to control potentially confounding environmental variables while experimentally manipulating those in question. We aimed to experimentally investigate the relative investment by mothers of these two types of egg resource depending on mate attractiveness. Importantly, looked at changes between successive clutches laid by the same female because large and repeatable inter-female differences in egg constituent allocation has been shown for most bird species studied to date.

The Australian zebra finch (*Taeniopygia guttata)* is an excellent avian model with which to investigate these questions as it will readily breed in a captive environment and male attractiveness can be manipulated using coloured leg bands (Burley et al. 1982; Hunt et al. 1997). Females find red bands attractive whilst green bands are an unattractive trait on a male. We used these known preferences to manipulate attractiveness of randomly assigned males to test whether this influenced female allocation of yolk resources. Specifically we investigated; (*i*) whether the quantity of androgens and antioxidants and/or the ratio between the two within each yolk varies with male attractiveness, and (*ii*) whether females varied the primary sex-ratio of offspring within a clutch or allocated resources in a sex-specific way as would be predicted for secondary sex ratio adjustment**.**

Hypothesis testing:

1. If females invest more resources in the offspring of attractive males (positive investment) we predict that eggs will contain higher levels of androgens and antioxidants. When considering the balance of the two resources within each yolk, we would also predict increased allocation of antioxidants relative to androgens, to compensate for their associated costs.
2. If females invest more resources when paired to unattractive males (compensatory investment) we would predict the exact opposite scenario. Yolks would contain higher androgens, antioxidants and larger ratio between the two in clutches laid for unattractive males.
3. Finally, if females adjusted resources in favour of sons of attractive males we expected these yolks to contain higher levels of androgens, antioxidants or a large ratio of the two resources (predictions summarised in table 1).

Hypothesis	Description of predicted response to male attractiveness manipulations	Upheld?
(1) Positive Investment	a) Higher concentrations of androgens and antioxidants with red banded males b) OR a larger ratio of antioxidants: androgens for red banded males	For daughters (DHTonly) For sons
(2) Compensatory Investment	a) Higher investment of androgens and antioxidants with green banded males b) Or a larger ratio of antioxidants: androgens for green banded males	For sons (DHT only) For daughters
(3) Trivers-Willard	a) Male biased sex-ratio for red banded males b) Higher concentrations of androgens and antioxidants for sons of red banded males c) OR a larger ratio of antioxidants: androgens for sons of red banded males	Yes More DHT for daughters Yes

Table 1: Outline of the investment hypotheses tested with predicted female investments of yolk androgens, antioxidants and sex allocation in response to male attractiveness treatments (red banded = attractive, green banded = unattractive).

Methods

Subjects and housing:

All birds were sourced from either local breeders or from the captive breeding population of zebra finches held at the University of St Andrews animal facility. Before being paired birds were housed in single sex aviaries and all were less than 6 months old. No birds had prior experience of red or green bands and this was their first ever breeding attempt.

We manipulated male attractiveness by randomly assigning males either red or green leg bands, one to each leg. There were 12 males in each treatment and each male was randomly assigned a female and placed in a breeding cage. All birds were measured for mass, tarsus length and fat deposition prior to pairing. This allowed two separate measures of condition; either a quantitative measure, the residuals of mass on skeletal size (tarsus length), or a qualitative measure of the amount of fat that had been stored in the furculum, which ranges from 0 to 5, such that 0 indicates no fat and 5 is convex and overflowing (Helms & Drury 1960). As female size and condition may influence egg resource allocation we confirmed that there was no difference in mass ($F_{1,23}$ = 2.184, p = 0.153) or fat score ($F_{1,23}$ = 0.548, p = 0.466) between females assigned to each treatment group.

All pairs were provided daily with *ad libitum* mixed seed (foreign finch mix by Haith's, Cleethorpes, Lincolnshire, UK), drinking water, cuttlefish and oyster-shell grit. This food was supplemented with Haith's egg biscuit and fresh spinach or cress every other day. All pairs were placed into individual breeding cages that were visually but not acoustically isolated from other birds. All cages contained a wooden nest cup and birds were provided with hay for use as nesting material. The lighting schedule was maintained at 14 hrs light : 10 hrs darkness and all artificial lights were full-spectrum Arcadia bird lamps (Arcadia Products plc, Redhill, UK). This is an important consideration since it has been shown that zebra finches require ultra-violet light to discriminate colour and make mate choice decisions (Bennett et al. 1996; Hunt et al. 1997).

Cross-over breeding design:

We checked all cages daily within 3 hours of the lights coming on so that all eggs could be immediately removed and replaced with a dummy egg (to prevent artificial

increase of clutch size). To ensure a full clutch had been taken, birds were left for 3 days after the last egg before being returned to the single sex aviaries. All females remained in the aviary for 2 weeks to ensure that when they were re-paired any stored sperm from the previous pairing would no longer be viable (Birkhead et al. 1989). Following this, females were returned to individual breeding cages and assigned a new mate, this time wearing the opposite band colour of the first mate. When males were repaired with a new partner they also wore the opposite band colour to the first breeding round. The experimental protocol was then repeated as described above.

All removed eggs were individually labelled using a non-toxic permanent marker pen and placed in an artificial incubator at 37.5 ^{o}C for 72 hours. This has been suggested to provide enough time for a large enough embryo to develop for genetic analysis without allowing yolk hormone or antioxidant levels to significantly change (Surai & Speake 1998; Gilbert et al. 2007). Once the incubation period was complete, all eggs were stored at -20 ^{o}C until further analysis. When a full clutch was frozen, eggs were split to remove the embryos which were placed in individual tubes and stored again at -20 ^{o}C for later genetic sexing. Whole yolks were then removed and separated from the albumin using the different thawing rates between yolk and albumin. All yolks were cut in half along the central line using a razor, weighed to 0.01 grams using a digital balance and each half placed in a separate labelled tube. One half of the yolk was stored at -20 ^{o}C and used for androgen extraction, the other half was used to extract antioxidants. As antioxidants are vulnerable to degradation they were stored at -70 ^{o}C.

Genetic sexing:

We extracted DNA from all embryos using the Puregene extraction method (Gentra Systems). Part of the W-linked avian CHD gene in females and its Z-linked homologue found in both sexes was amplified using the polymerase chain reaction (PCR) using specific zebra finch primers ZF1 and ZF2 (Rutstein et al 2004c). PCR's contained 10-500 ng of genomic DNA, 0.2 µl of 8mM dNTP, 0.2 µl each of 50 mM primers ZF1 and ZF2, 0.5 units of Taq polymerase (Bioline), 0.6 µl of 50 mM $MgCl_2$, 1 µl Bioline 10 x NH_4 reaction buffer which gave a total volume of 10 µl. The PCR products were separated on a 3% agarose gels at 50 V and visualised using ethidium bromide. Females had two bands at 350 and 384 bp and males one at 350 bp.

Androgen extraction assay:

Total yolk androgens were extracted using a solid-phase extraction using Sep-Pak C18 cartridges (Waters Ltd, Elstree, Hertfordshire, UK). Cartridges were first primed with methanol and then washed with MilliQ water. Frozen half-yolks were homogenized in 500 µl of water and this solution was passed through a cartridge. Samples were then washed with acetone and finally eluted in methanol. The methanol was evaporated off and the dried sample stored in a -20 ^{o}C freezer. Testosterone (T) and dihydrotestosterone (DHT) concentrations were measured using Diagnostic Systems radioimmunoassay kits (DSL 4000 and DSL 9600). Before DHT could be measured the samples required an extra step to oxidise T, using the potassium permanganate buffer provided. Therefore, the T assay initially gave the combined concentration of DHT + T and required back-calculation to give the actual amount of T in a sample. All samples were assayed using competitive binding RIA and were run in duplicate, and concentrations were compared to standard curves. Cross reactivity of the T antiserum with DHT is 5.8% and the DHT antiserum with T (following further extraction) is 0.02%. Cross-reactivity with other steroids is minimal for both assays (<5%) (figures provided by DSL). The intra-assay coefficient of variation was 3.3% ± 0.95 SE for T and 0.9 ± 0.18 SE for DHT. All yolks from the same mother were run within the same assay.

Antioxidant extraction and assay:

Frozen half yolks were homogenized in an equal volume of water and an aliquot of 100 µl (50 mg) was mixed with 0.7 ml of 5% NaCl and 1 ml ethanol. An internal standard (canthaxanthin) was added and the mixture vortexed for 30s. 2ml of hexane were added and the solution homogenized for 1 min. The hexane phase that contained the lipid-soluble carotenoids and vitamins was collected. This extraction was repeated and the collected supernatant combined, dried under a vacuum and re-suspended in 600 µl of 1:1 methanol/dichloromethane. The carotenoid and tocopherol concentrations were determined by reverse-phase HPLC using a Phenomenex Synergi 4µm Hydro-RP 80A (2×250 mm) column with a mobile phase of acetonitrile:water (97.5:2.5) and ethyl acetate:water (97.5:2.5) in a gradient elution at a constant flow rate of 0.2 ml/min for 56 min. Peaks were detected by a Spectra System UV6000 LP photodiode array detector, and peak areas were integrated at 445–455 nm (carotenoids) and 290–300 nm (tocopherols) wavelength ranges with

Xcalibur software. For our analysis we focused on the total carotenoid (predominantly Lutein and Anhydrolutein) and α-tocopherol levels.

Statistics:

General linear mixed models (PROC MIXED) were performed in SAS version 9.1 (SAS Institute Inc, Cary, NC, USA) to analyse variation in yolk androgens (T and DHT) and antioxidants (total carotenoids and vitamin E) – the response variables. Female identity was entered as a random factor, fathers' band colour as a fixed effect and eggs within a clutch as a repeated measure. Explanatory variables initially entered into GLMs were: father's band colour, mother's residual condition or mother's fat score (in separate models), the laying order of eggs within the clutch, sex of the embryo and breeding round (first or second clutch). Two-way interactions between all variables and father's band colour were also initially entered and then a backwards stepwise procedure followed, removing non-significant variables ($p>0.05$) from the model starting with the least significant interactions. The Satterthwaite approximation was used to calculate denominator degrees of freedom (Littell et al, 2004). We then analysed sex-ratio as the response variable by fitting a generalized linear mixed model (PROC GLIMMIX) with a binomial error distribution and logit link function. Female identity was entered as a random factor and all the same explanatory variables and interactions as were entered for the androgen and antioxidant models. Tables of the model output that include values for all variables for the initial model and the final models are presented in appendices. All other analyses were performed in SPSS version 16 (SPSS Inc, Chicago, Illinois, USA).

Results

48 clutches were laid and 173 yolks assayed for androgen and antioxidant content. Due to some eggs failing to develop an embryo, 133 offspring were sexed (77 male and 56 female). Clutch sizes varied between two and six eggs. Egg mass significantly increased with the laying order within the clutch, with the last laid eggs being the heaviest ($F_{1,171} = 7.85$, $P = 0.006$). However, there was no such pattern for yolk mass within clutches ($F_{1,171} = 2.30$, $P = 0.13$; appendix 1). As yolk mass was consistent across the clutch we did not incorporate this to calculate total qualities of androgens or antioxidants in the egg since it added no further information and could potentially increase measurement error. All analyses were initially conducted on total hormone and antioxidant amounts in the yolks and virtually identical results obtained. To reduce repetition and reduce error all the following data presented are concentrations of androgens and antioxidants per unit mass of the yolk. All data were natural log transformed to achieve normal distributions.

Yolk androgens

The predominant androgen in yolks was testosterone (T) (mean concentration = 21.25 pg/g ± 0.98 SE) compared to dihydrotestosterone (DHT) (mean concentration = 3.49 pg/g ±0.11 SE) but the concentrations were highly positively correlated within each yolk (Pearson's correlation = 0.699, $p<0.0001$). Both T and DHT concentrations significantly decreased across the laying order (GLMM; T: $F_{5, 128} = 10.75$, $P < 0.0001$, DHT: $F_{5,128} = 2.86$, $P = 0.018$) and there was no evidence to suggest that this pattern with laying order was influenced by male attractiveness (T*Ring colour: $F_{5, 121} = 1.47$, $P = 0.20$, DHT*Ring colour: $F_{5,122} = 0.52$, $P = 0.76$: figure 1). Mother's fat score was found to significantly negatively influence yolk T and DHT concentrations (T: $F_{1, 44.7} = 7.84$ $P = 0.01$, DHT: $F_{1,39.3} = 8.29$ $P = 0.01$). Females with a higher fat score deposited lower levels of both T and DHT in their yolks. There was significantly lower yolk T concentration in eggs that had no embryo compared to male and female eggs ($F_{2,142} = 3.57$ $P = 0.03$), but T concentration did not vary with the sex of the embryo (Tukey post-hoc tests; son:daughter $t_{128} = 0.31$, adj. $p = 0.95$, son:no embryo $t_{140} = -2.59$, adj. $p = 0.03$, daughter:no embryo $t_{143} = -2.34$, adj. $p = 0.05$). There was

a significant interaction between embryo sex and father's band colour for the concentration of DHT in the egg yolk ($F_{2,149} = 4.49$, $P = 0.01$). Daughters received higher levels of DHT when fathered by an attractive male compared to an unattractive male, and the opposite pattern was shown for sons. There was no such difference in male band colour between eggs that contained no embryo (figure 2). For full models and values of non-significant variables that were removed from the final models see appendices 2 & 3.

Yolk antioxidants

Yolks contained more total carotenoids (mean concentration = 56.02 µg/g ± 2.95 SE) compared to vitamin E (mean concentration = 29.8 µg/g ± 1.32 SE) and the two were highly correlated within eggs (Pearson's correlation; n = 173, r = 0.518, p < 0.0001). There was a significant decrease in both total carotenoid and vitamin E concentrations across the laying order (GLMM: vitamin E: $F_{5, 101} = 3.51$, $P = 0.006$; carotenoids: $F_{5, 128} = 10.82$, $P < 0.0001$; figure 3). Total carotenoid concentration was found to significantly vary with residual female condition ($F_{1, 46.7} = 10.92$ $P = 0.002$; appendix 4); females that had higher relative condition at the start of each breeding round deposited higher levels of carotenoids in their yolk. All other values were non-significant and removed from final models (see appendices 5 & 6 for full details).

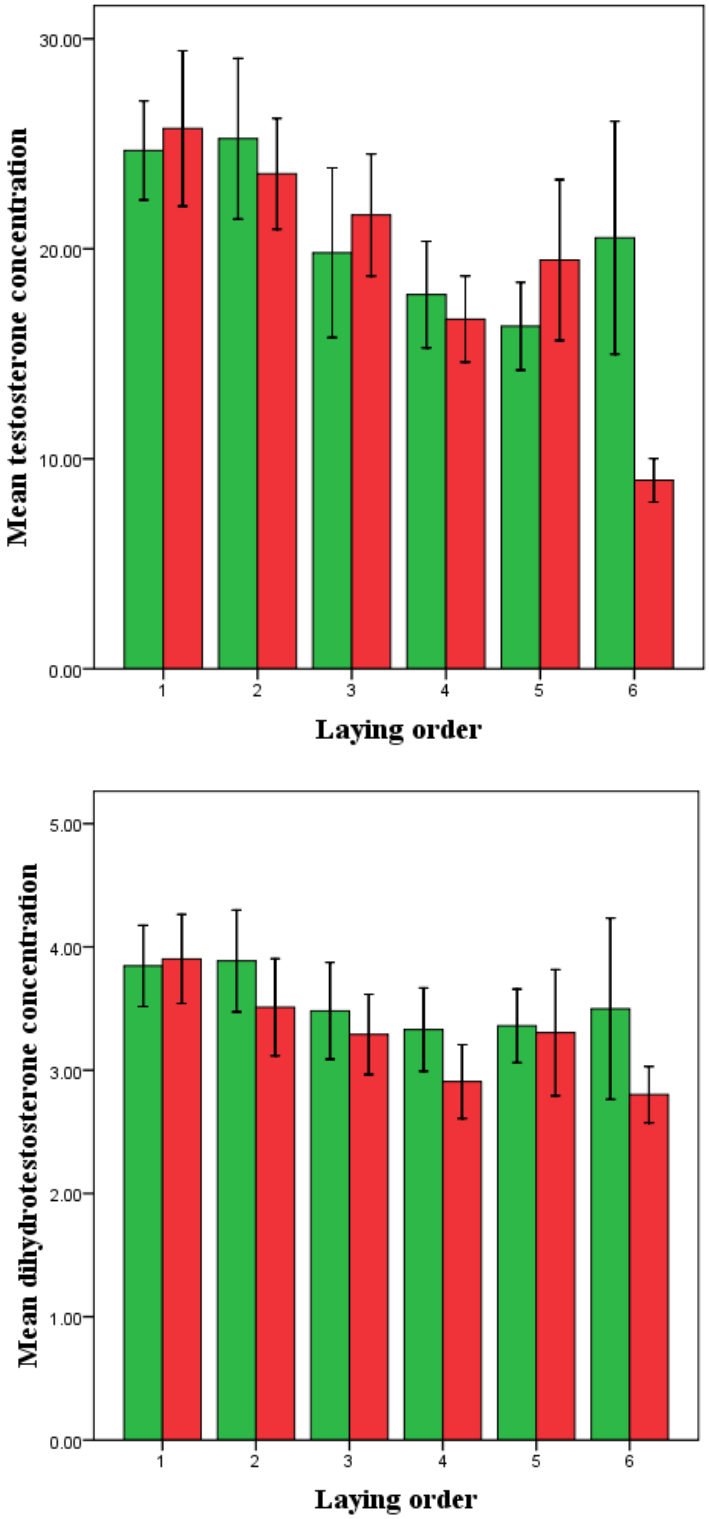


Figure 1: Mean T and DHT concentrations in pg/mg (±SE) across the laying order from the first laid to sixth laid eggs for clutches laid for attractive (red banded) and unattractive (green banded) males.

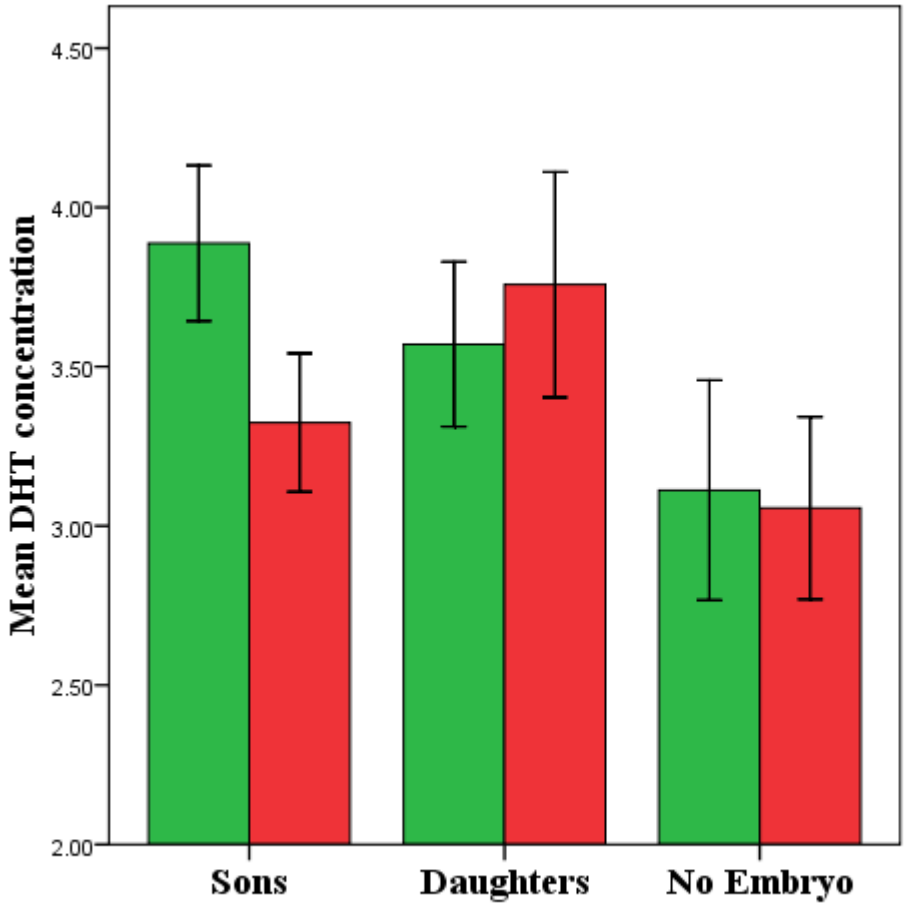
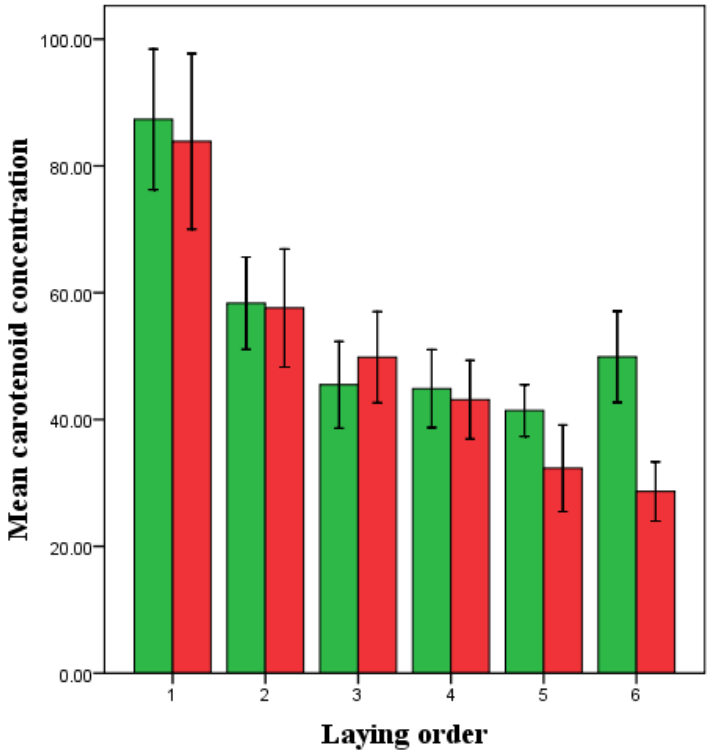


Figure 2: Mean DHT concentration natural log transformed pg/mg (±SE) in yolks of sons, daughters and eggs with no developed embryo laid for attractive (red banded) and unattractive (green banded) males.



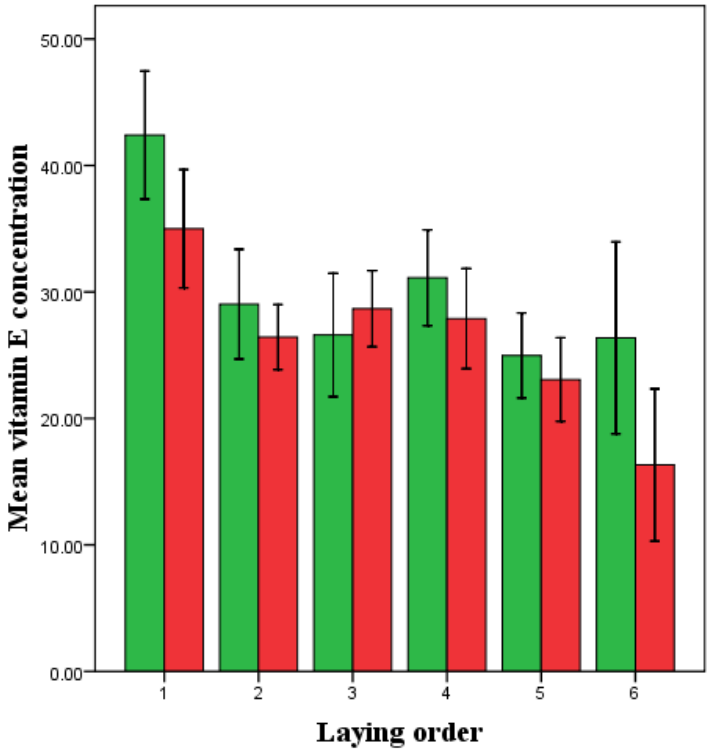


Figure 3: Mean total carotenoid and vitamin E concentrations (ug/g) (±SE) across the laying order of each clutch from first laid to sixth laid eggs for attractive (red banded) and unattractive (green banded) males.

Ratio of yolk antioxidants/androgens

There was no direct correlation between the total concentrations of antioxidant and androgens measured in each individual egg yolk (Pearson's correlation; n = 173, r = 0.122, p = 0.11; appendix 7). To test whether the ratio of yolk androgen to antioxidants within yolks varied between individual eggs we calculated the ratio of total androgens measured (combined T and DHT) with total antioxidants measured (combined vitamin E and carotenoids). We calculated the antioxidant/androgen ratio by dividing the total antioxidant concentration (in µg/g) by the total androgen concentration (pg/mg). The resulting numbers were natural log transformed to give a normal distribution for further analysis. As the previous data showed no variation in yolk constituents within eggs lacking an embryo we only considered eggs containing embryos for this analysis.

The antioxidant: androgen ratio significantly differed depending on offspring sex and the father's band colour (GLMM: Sex*Band colour: $F_{1,93.6} = 6.42$, P = 0.01). Mothers allocated a smaller concentration of androgen per antioxidant for female eggs when paired to unattractive males compared to attractive males. This pattern was reversed for sons, with a larger amount of androgen per antioxidant when fathered by attractive males compared to unattractive (see figure 4). We also found that the ratio of the constituents was significantly influenced by mother's fat score ($F_{1,36.8}= 9.81$, P = 0.003) and condition ($F_{1,27.7}= 8.49$, P = 0.01). Mothers with a higher fat score and better condition had a larger antioxidant: androgen ratio within their eggs. All other variables were non-significant and removed from the final model (appendix 8).

Primary sex-ratio

There was a significant interaction between maternal condition and paternal band colour for the sex-ratio of a clutch (GLIMMIX; $F_{1,109}= 7.73$, P = 0.01; figure 5). When paired with red-banded males all females had a male biased brood, but this was more male biased for females in higher condition. However, females paired to green-banded males laid male biased broods when in poor condition and female biased broods when in good condition. All other variables tested were not found to significantly influence sex-ratio and where removed from the final model (see appendix 9).

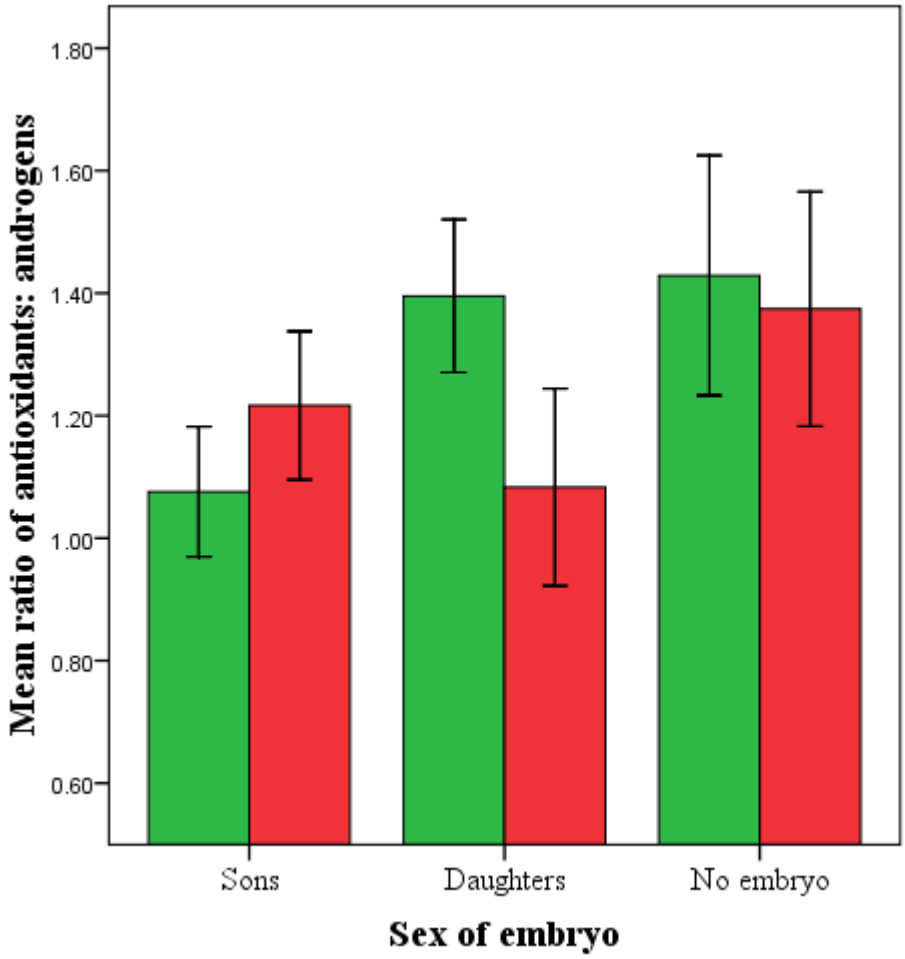


Figure 4. Mean ratio of total yolk antioxidants: total yolk androgens, ln transformed (± SE) within eggs laid for attractive (red banded) and unattractive (green banded) males.

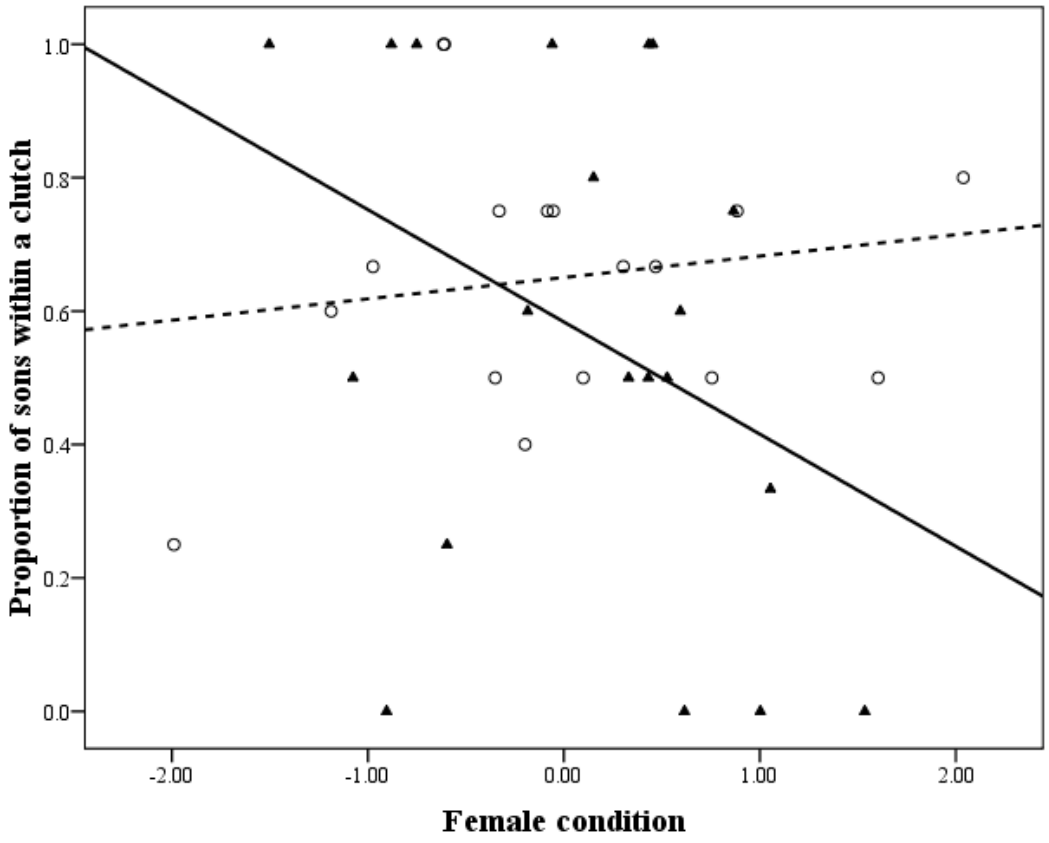


Figure 5: Proportion of sons within each clutch against female condition (calculated from the residuals of mass: tarsus) and male band colour. Black triangles represent clutches laid for green banded males (solid line of best fit) and white circles represent red banded males (dotted line of best fit). No embryo's developed in 19 eggs laid for green banded males and 21 eggs for red banded males. Modal clutch size for both treatment groups = 5 eggs.

Discussion

The aims of our study were to test whether mothers can manipulate the allocation of yolk antioxidants and/or androgens as would be predicted by the positive investment, compensatory investment or Trivers-Willard hypotheses (see table 1). Our results support the Trivers-Willard theory of sex allocation, that mothers will alter resource allocation in a good environment (or attractive mate) towards the offspring with the most variable reproductive success (Trivers & Willard 1973). In most species this predicts resource allocation in favour of sons, as has previously demonstrated in the earlier studies of the zebra finch (Burley 1981; 1986).

Yolk androgens & antioxidants

We found that mothers allocated a smaller ratio of androgen to antioxidant to daughters when paired to green ringed males compared to red ringed males. This pattern was reversed for sons, with a larger ratio of androgen to antioxidant when fathered by red ringed (attractive) compared to green ringed (unattractive) males. If relatively large quantities of antioxidants are required to balance the side-effects of androgens (such as reduced immune function and oxidative stress caused by growth and development, Royle et al. 2001), this ratio of resources would be more beneficial to the developing offspring than a small ratio.

However, it is important to note that it is only when considering the ratio of antioxidants to androgens that we found this pattern. If we looked solely at the allocation of androgens, we found that females allocate more DHT into the eggs of daughters of attractive males compared to those with an unattractive father. The opposite allocation was found for sons, showing higher concentrations of DHT if they had an unattractive father compared to an attractive one. This could either been seen as supporting the compensatory investment hypothesis or the positive investment hypothesis, depending on which sex of offspring is being considered (see table 1). Neither pattern of allocation could be found if antioxidant concentrations were considered separately.

More direct manipulation experiments are required to conclusively understand what ratio of antioxidants to androgens in the yolk is optimal for developing offspring. Current evidence supports the idea that the higher levels of yolk androgen

are beneficial for growth, development and competitive ability of chicks (Schwabl 1996; Lipar & Ketterson 2000; Eising & Groothius 2003). However, studies that have specifically tested the influence of increased yolk androgen concentration on zebra finch chicks found that these effects depended upon the sex of the chicks (von Engelhardt et al. 2006; Tobler & Sandell 2009). von Engelhardt et al. (2006) found that daughters reaped greater rewards than sons from increased yolk T concentrations, increasing both growth and begging behaviour relative to their normally dominant brothers. Interestingly, a more recent study that analysed blood plasma of nestlings that had hatched from injected eggs found that male offspring suffered lower antioxidant capacity when T levels were elevated (Tobler & Sandell 2009). Therefore, the increased androgen concentrations in the yolks of the daughters of attractive males in our study could be seen as a strategy by mothers to increase the daughters' chances of survival in these clutches by improving their rates of growth and begging (positive investment). If such effects cause female offspring of attractive males to survive better than their brothers, it would result in a female-biased secondary sex ratio. However, the opposite effect of mate attractiveness on sex-specific offspring survival might be expected if we consider the antioxidant : androgen ratio in the yolk. Daughters received a lower ratio of antioxidants to androgens when they had an attractive rather than an unattractive father. Consequently, these daughters may show faster development, but suffer the associated oxidative stress due to elevated yolk androgens, without the benefit of proportionally elevated antioxidants. Further support for this idea can be found in a recent study that found an increase in dietary antioxidants was required to mitigate detrimental effects of elevated yolk T levels (Cucco et al. 2008). Therefore, the sons of attractive males that received a higher antioxidant: androgen ratio could be benefiting more than their sisters, supporting both the Trivers-Willard and positive investment hypotheses.

Primary sex-ratio:

Females paired to red banded (attractive) males had male-biased within-clutch primary sex ratios. However, the sex-ratio was also influenced by the condition of the laying female. Females in poor condition had male biased broods for both attractive and unattractive males. Whereas good condition females followed the predictions of sex-allocation, by laying clutches with male-biased broods for attractive males and female biased broods for unattractive males. These data suggest that the allocation

decisions made by a female depend not only on her external environment but also her own condition, which fits predictions made by a recently published theoretical model (Harris & Uller 2009). The authors found that females are to likely follow a compensatory investment strategy when they are older or in poor condition, suggesting allocation strategies are flexible within species and individuals.

Interpreting data & future directions

Since eggs were not allowed to hatch, we were unable to determine the fitness consequences for daughters and sons attributed to the relative yolk deposition of androgens and antioxidants. Nonetheless, we would stress that to regard elevated levels of yolk androgens as having positive fitness consequences for developing offspring may be an over-simplification. Fitness consequences will generally depend on the context, which judging by our results, could include the sex, laying order and quality of the individual offspring as well as the amounts of other yolk resources such as antioxidants. Very few studies have investigated the inter-relationships between yolk constituents and none via direct manipulation of qualities within the yolk. Two studies looking at the balance of androgens and antioxidants in black-headed gulls have shown patterns of increased androgen and decreased antioxidant concentrations across the clutch (Royle et al. 2001; Groothuis et al. 2006). However, each study interprets this pattern of resource deposition over the clutch in a different way. Royle et al. (2001) suggest that mothers deposit low levels of antioxidant and high levels of androgen in last laid eggs as a form of adaptive brood reduction in case of low food availability. The alternative explanation put forward by Groothuis et al. (2006) is that more androgens relative to antioxidants could be seen as beneficial for these last laid eggs since the immunomodulatory effects of testosterone would redirect the energy required to developing an immune system to focus purely on growth and competitive behaviour. Under this hypothesis our results could be interpreted in the opposite direction, whereby the decreased antioxidant: androgen ratio in daughters' eggs could be increasing their chance of competing and surviving.

The relative proportions of yolk androgens and antioxidants also vary between species, with a positive association found in house finches (*Carpodacus mexicanus*) (Navara et al. 2006) and no association at all in the eggs of either barn swallows *Hirundo rustica* (Safran et al. 2008) or collared flycatchers *Ficedula albicollis* (Hargitai et al. 2009). All of these studies used wild populations of birds, so the scope

to experimentally control or manipulate environmental variables was limited. To the best of our knowledge this is the first study to test for the interaction between yolk androgen and antioxidants in a controlled laboratory experiment where manipulating mate attractiveness was possible. The within-female cross-over breeding design controlled for variation among females. Our data suggest that females are capable of adjusting yolk constituents and can subtly change the balance of these within each yolk dependent on offspring sex and on the attractiveness of her mate. We do not yet understand the fitness consequences of the ratio of constituents to developing offspring. Experiments that directly manipulate the relative levels of yolk constituents and then monitor the performance and phenotype of the developing offspring are needed to examine this further. There are many conflicting theories predicting the direction in which females should respond to changing environments, however, it is likely that mothers are flexible in order to rapidly and appropriately respond in a variable environment. Our data also suggests that further investigation into the influence of female condition on resource allocation strategies will both advance our understanding of this topic and aid in interpreting the discrepancies between studies (Bolund et al. 2009; Harris & Uller 2009). The mechanism behind female allocation of resources to avian yolks is also currently unknown (Groothius & Schawbl 2008). This gap in our knowledge needs to be filled before full interpretation of data such as ours can be made.

Chapter 3

Artificial ornaments manipulate intrinsic male quality in wild caught zebra finches (*Taeniopygia guttata*)

Emma Pariser, Mylene Mariette, Nina Svedin & Simon Griffith

I collected 70% of data and carried out 100 % of analysis and write-up. MM conducted one round of mate choice trials in experiment 1 and NS conducted all trials in experiment 2. I received comments and feedback on drafts from MM, NS and SG.

Abstract

The addition of red and green colour bands is a commonly used method for manipulating male attractiveness in the zebra finch (*Taeniopygia guttata*), providing insight into the study of maternal investment and sexual selection. The addition of artificial ornaments has been assumed to manipulate a female's perception of the male, rather than affecting intrinsic qualities of the male himself. Here however we reveal that the artificial band colour worn by a male changes his body mass, condition and courtship display rate. Males wearing red colour bands in aviaries prior to mate choice trials had a significantly higher song rate during trials than those wearing green colour bands, alongside a significant increase in mass change and condition. Male song rate was found to significantly correlate with female preference alongside a female preference for red banded males. However, male song rate in turn increased when female response was positive, suggesting a social feedback between the interacting birds. Our data suggest the presence of socially mediated feedback mechanisms whereby the artificial increase in attractiveness or dominance of a male directly affects other aspects of his attractiveness. Therefore, housing birds in social groups whilst manipulating attractiveness can directly influence other male qualities, and should be accounted for by future studies.

Introduction

The ability to manipulate the attractiveness of an individual has been a useful tool in behavioural ecology, allowing powerful and highly controlled tests of maternal allocation strategies and sexual selection theory. Methods used to achieve these manipulations vary depending on the organism, from adjusting tail length in widowbirds (*Euplectes progne*) (Andersson 1982), and photographic facial reconstructions in humans (Swaddle and Cuthill 1995) to removing ultra-violet reflectance in blue tits (*Cyanistes caeruleus*) (Sheldon et al. 1999). However, rather than manipulating an existing trait, the addition of a purely artificial trait is appealing

because its influence should be purely superficial and easily reversible. For these reasons, the discovery that female zebra finches (*Taeniopygia guttata*) altered their behaviour depending on the colour of leg bands worn by males for identification purposes (Burley et al. 1982), presented a new research opportunity. Repeated mate choice experiments have shown that females prefer males wearing red leg bands and have an aversion to males wearing light green leg bands (Burley et al. 1982; Burley 1988b; Hunt et al. 1997; but see Jennions 1998). This manipulation has subsequently become one of the most replicated in behavioural ecology (Burley 1985, 1986a, b, 1988b; Bennett et al.1996; Gil et al. 1999; Rutstein et al. 2004, 2005; Gilbert et al. 2006; Williamson et al. 2006). Although the mechanisms behind these preferences are unknown, it has been hypothesized to enhance the natural preference for red male colouration (potentially signalling quality), to aid sexual differentiation between males and females, or simply reflect an arbitrary choice (Burley 1986b). Importantly, studies using colour band manipulations assume that the ornament does not influence any other aspect of male quality, and that female preference for individuals wearing bands would expire following their removal.

The first suggestion that colour bands had multiple effects on males wearing them was in an early study demonstrating a higher mortality of green banded birds compared to red banded birds during aviary breeding experiments (Burley 1985). Competition between males was ruled out as an explanation and instead the main hypothesis focused on the idea that red banded males were less physically stressed during reproduction than the green banded males (supporting the later published differential allocation hypothesis; Burley 1988a). Interestingly, in a later study, Burley (1986a) showed that colour bands did appear to influence male-male interactions. This hypothesis has since been experimentally tested and confirmed; Cuthill et al. (1997) found that red-banded males dominated green-banded males over access to food. This experiment investigated both the immediate effect of colour bands on interacting birds (by monitoring small groups over the course of one day), and also the prolonged effect on weight change over a ten day period. Cuthill et al. (1997) concluded that colour bands influenced male dominance and explained variations in daily changes in fat deposition and weight. Since this study, however, there have been no investigations into how this variation in dominance may affect males in the long term, and specifically whether male dominance may influence female mate preferences (as suggested in the review by Collins and ten Cate 1996).

Investigating the influence of colour bands on male quality is essential for studies that have used artificial ornaments as a means to manipulate male attractiveness in sex allocation and maternal investment strategies (Burley 1986a, 1988a; Rutstein et al. 2005). Fully understanding the influence of adding artificial ornaments is crucial because interpretations may fail to account for the possible confounding effects of unplanned manipulations of paternal quality. This is especially important in species with bi-parental care, such as the zebra finch. An awareness of the possible confounding effects of colour bands will aid our interpretation of the studies published to date and the design of future studies.

Our aims with this study were threefold. First, to investigate the long term effects of colour bands worn by males in a social environment, specifically whether colour would influence the wearer's intrinsic condition and/or attractiveness to females. Second, to confirm that a population of females also showed mate choice preferences based on colour bands, and finally, to conduct all experiments on wild caught birds thereby removing any effects of domestication. Discrepancies between studies have led to increasing concern about the influence of inbreeding and possible artificial selection on domesticated finches (Forstmeier et al. 2007; Rutstein et al. 2007). This, combined with the fact that colour band preferences have only been tested once on wild caught birds (Burley 1988b), highlights the importance of confirming these data in a wild-caught population.

We predicted that if red bands confer dominance advantages to the wearer, over green and neutrally banded males; this may result in red banded males being in significantly better condition (physical health and expression of condition-dependent secondary sexual traits) after an extended period of time. Any consistent difference in the variation of condition or social dominance among males wearing different coloured bands is likely to affect their display rate, which in turn would influence their attractiveness to females in mate choice trials.

Methods

Subjects and housing

Adult birds (67 males and 57 females) were caught in Sturt National Park, far-western New South Wales, Australia (under licence from the department of environment, climate change and water, NSW government). Birds were then transported to Fowler's Gap Arid Zone Research Station (31^{o} 05'S, 142 ^{o} 43'E) where they were housed in one of three single sex aviaries (2m x 3m x 2m). All birds were fitted with individually numbered white identification bands and males were sequentially assigned to one of three treatment groups, and given either an extra red or green colour band on each leg (A C Hughes, Middlesex, UK) or no extra colour band. Birds were housed in these groups from September 2007 until January 2008 (one aviary housed 11 males of each treatment, the other had 12 green-banded males and 11 males in each of the other two treatments).

At the end of this period, all birds were transported to Macquarie University (Sydney) and placed into single sex cages in groups of up to 4 birds per cage (all cage mates were from the same aviary group). Each male cage included at least one individual from each of the three treatment groups. After a two week acclimatization period the first mate choice trails were performed (Experiment 1). Following completion of these trials, all birds were introduced into one of two large outdoor aviaries at Macquarie University (10m x 8m x 2m). At this stage, groups were mixed in equal numbers of males and females and provided with nest-boxes, allowing birds to pair and breed. We also reduced the study to two treatment groups, red-banded birds or green-banded birds (i.e. randomly assigning all birds with only a white identity band, to either red or green treatments). Birds then remained in these groups (with bands on) from March to November 2008, when the birds were once again returned to two single sex groups (within the aviaries) for one month, prior to repeating the mate choice trials in January 2009 (Experiment 2). Over this 18 month period, natural mortality decreased the sample sizes for each experiment (see Figure 1 for details).

Birds were provided with ad libitum mixed seed (Golden Cob, Sydney, Australia), cuttlefish, oystershell grit, spinach and water. While in Sydney, birds were

also given sprouted green seed daily. Aviaries were outdoor and subject to daily changes of natural light and temperature. When housed in cages, a constant temperature of (28^{o}C) was maintained, with a 14:10 hour light:dark schedule.

Morphological measurements

All birds were measured for tarsus length (0.01 mm) and weight (0.1g) to calculate condition scores (from the residuals of mass on tarsus). Measurements were taken at three stages; 1) when birds were first caught, 2) following the 5 month period in single sex aviaries, and 3) following the 8 month period in mixed flock aviaries.

Mate choice protocol

No-choice trials were used to assess mate preferences and courtship behaviour by scoring observations over a 5 minute interaction period (Forstmeier and Birkhead 2004; Rutstein et al. 2007). This procedure involved placing a male and female on either side of a removable partition dividing the test cage (750mm x 480mm x 400mm). After a 2 minute acclimatization period, the partition was removed and both male and female behaviour was directly observed and scored from behind a screen. Female behaviour was either scored as positive (+1) if witnessed approaching the male whilst performing ritualized hops, beak swipes or tail quivers, negative (-1) (if witnessed fleeing from the male or aggressive pecks and chases, or neutral (0) if none of the previous responses were observed. As negative responses were rare, and in effect a neutral response is a negative mate choice, these two categories were pooled for all statistical analysis. For males, the total amount of time (sec) spent singing within the 5 minute trial was also recorded using a handheld stopwatch.

Experiment 1: Colour band trials

We conducted the first set of mate choice trials to simultaneously test female preference for band colours, as well as the influence of band colour on male courtship behaviour. To test this, each male took part in four trials, with a different random female on each occasion. In the first trial, males wore the band colour that they had been previously wearing for 5 months in the aviary. For the second trial, they were assigned the opposing band colour to their aviary colour (i.e. red were switched to green and vice versa; males wearing only white identity bands were changed to another possible control – orange bands on both legs). The third trial consisted of half

of the males wearing their aviary colour and half wearing the opposing option. This was reversed for the fourth trial. Thus, following four trials, each male had been viewed by four different females; twice wearing the aviary band colour, and twice wearing the opposing colour. 58 males were tested using 51 females (if an individual female was tested in more than 4 trials, only the first four trials were considered when analyzing female preference). This gave a total of 232 trials to test male behaviour and 204 trials for female behaviour.

Experiment 2 – No leg bands trials

Approximately 12 months after Experiment 1, another round of mate choice trials were conducted using the same individuals with an identical experimental set-up. This experiment was designed to replicate the first mate choice experiment while controlling for female response to band colours during trials. So for all trials males had their leg bands removed (including white identity bands). Again, males were tested four times with four different randomly assigned females (making sure each male-female trial dyad were naïve to each other). For these trials, a total of 30 males (15 red and 15 green) were tested with 36 females. If males were included in more than 4 trials, only the first four were analysed with respect to male behaviour. Thus, a total of 120 trials were analysed to test male behaviour and 144 trials for female choice.

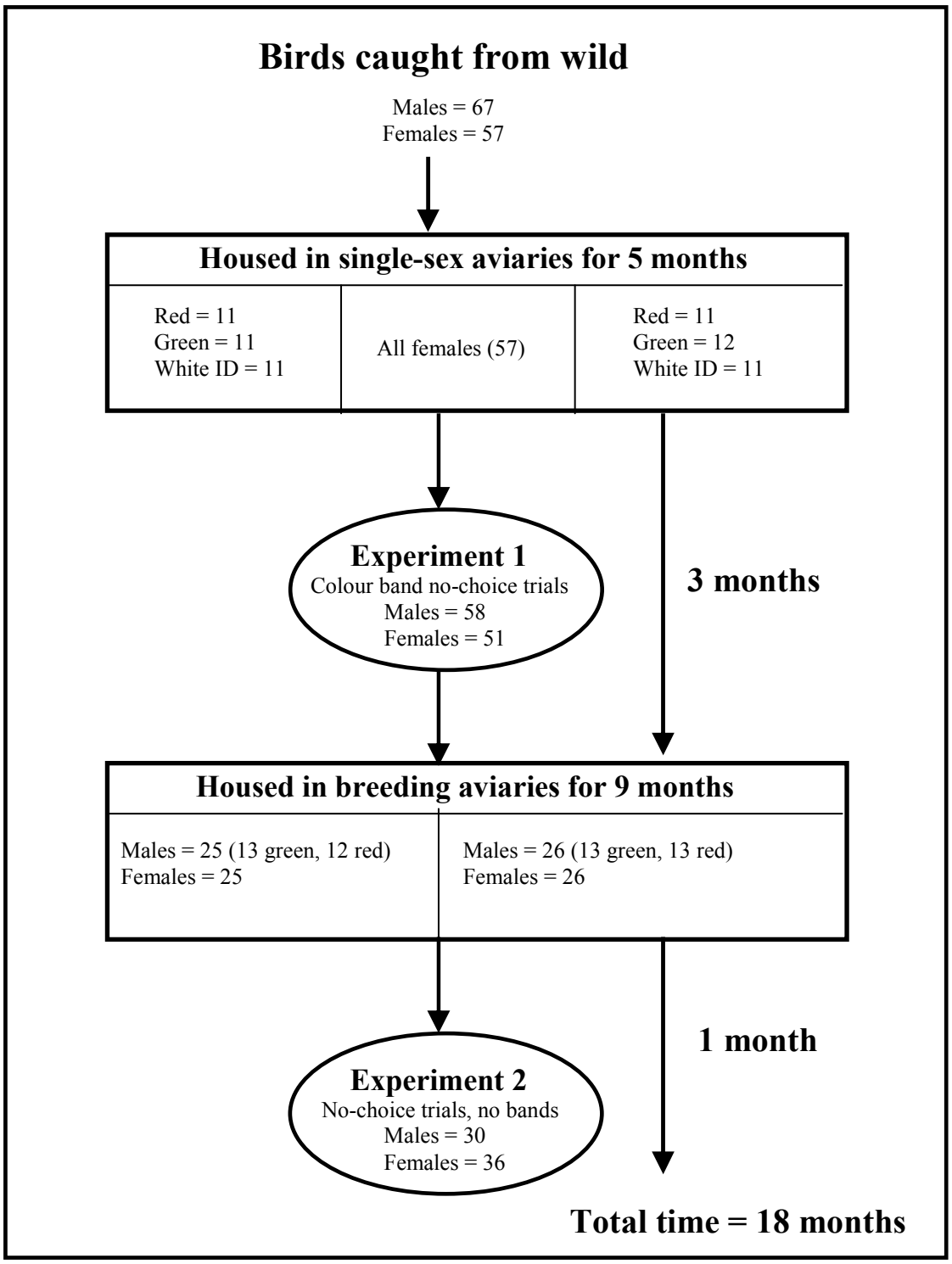


Figure 1: Flow-diagram showing the timeline of experiments, with sample sizes at each stage.

Statistical Analyses

For both experiments, male song rate was analysed using a General Linear Mixed Model (GLMM) in the SAS system for Windows version 9.1 (SAS Institute Inc., Cary, NC, USA). Explanatory variables entered were: trial band colour worn by the male, the band colour that had been worn in the aviary, male condition, female condition, female response behaviour and trial number. Female identity was entered as a random factor and male identity as a repeated measure. For both experiments, song rate was transformed to reduce deviation from normality; for Experiment 1 the data were transformed as y' = (y + 1)**0.0723 and for experiment 2 as y'= (y + 1)**0.3399 (calculated using the Box-Cox function in R 2.4.1(Box & Cox, 1964)). This dataset consisted only of males that had courted a female in at least one of the trials; 11 non-responsive individuals were removed in the first experiment (2 green banded, 4 red banded and 5 white ID band only males).

Female response was analysed by fitting a Generalized Linear Mixed Model (GLIMMIX) with a binomial error distribution and logit link function in SAS. Explanatory variables entered were: trial band colour worn by the male, the band colour that had been worn in the aviary, male condition, song rate, female condition and trial number. Male identity was entered as a random factor and female identity as a repeated measure.

For all models, non-significant variables were removed sequentially, in a backwards stepwise procedure following the removal of the least significant term ($P<0.1$). Repeatability of male song rate was calculated from variance components of a one-way ANOVA (Lessells and Boag 1987). All other statistical tests were performed using SPSS version 16 for windows (SPSS Inc, Chicago, USA) and all tests reported are two tailed. All data were checked for normality using Kolmogorov-Smirnov tests and transformed if necessary.

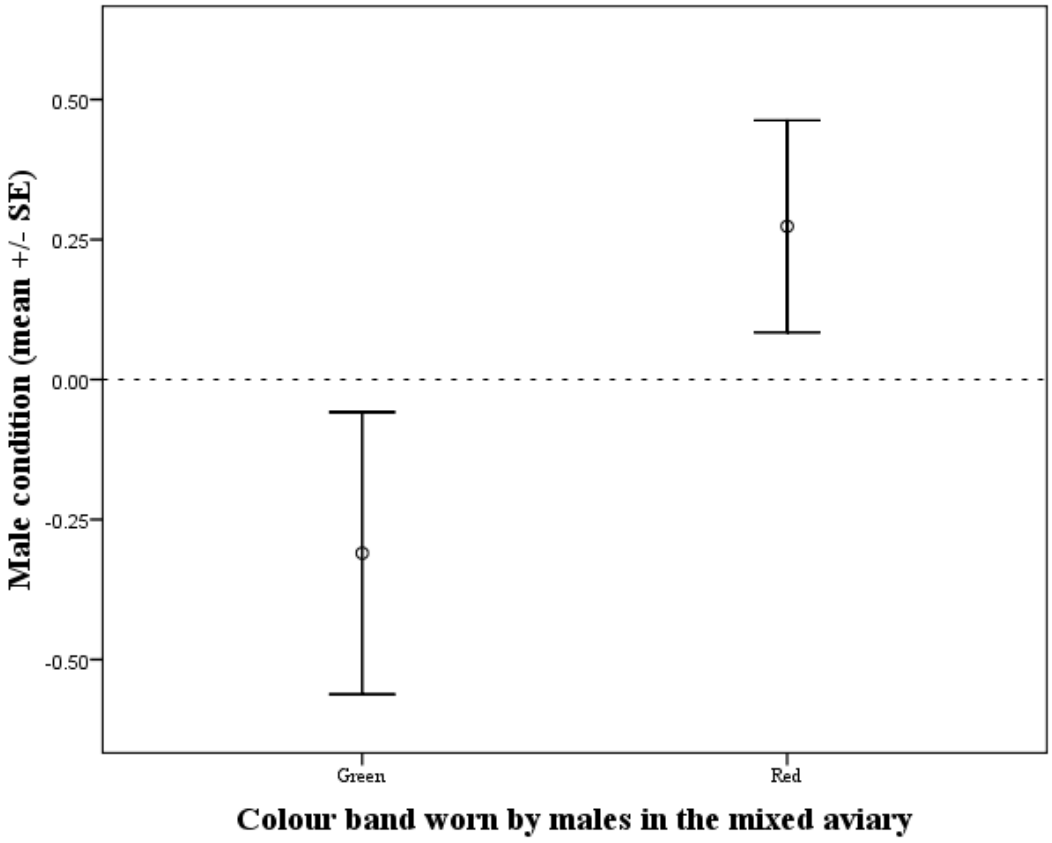


Figure 1: Mean (± SE) of male condition (from residuals mass:tarsus) for males that had been wearing red or green bands for 8 months in mixed group aviaries.

Results

Male morphology and condition

When first assigned to the treatment groups there was no significant difference between males in either weight (ANOVA: $F_{2,44}$ = 1.105, P = 0.34) or relative condition ($F_{2,44}$ = 1.114, P = 0.34). This result remained following five months housing in male-only groups (ANOVA: Weight $F_{2,44}$ = 0.341, P = 0.71; Condition $F_{2,44}$ = 0.234, P = 0.79). However, when the remaining 32 males were re-measured following the eight month period housed in mixed-sex groups, red-banded males (n=17, mean = 0.27, SE = 0.19) were in relatively higher condition than green-banded males (n=15, mean = -0.31, SE = 0.25) (T-test; $t_{1,30}$ = 2.023, p = 0.052; Figure 1). For the surviving 32 males that were included in all analyses, percentage weight change was calculated for each of the two treatment periods (male only aviaries and mixed aviaries) using the equation ((final weight – initial weight)/ initial weight)*100. After the time spent in male only aviaries, in which males had been split into 3 treatment groups, there was no significant difference in the average percentage weight change (ANOVA: Weight $F_{2,29}$ = 0.762, P = 0.48). On average all males increased in weight while in the male only aviaries for all treatment groups (red: n = 8, mean = 9.13, sd = 9.00; green: n = 10, mean = 5.45, sd = 5.82; white identity band: n = 14, mean = 5.62, sd = 6.77).

No-choice trials

Male song rate:

In Experiment 1, the strongest predictor of male song rate was the colour of the bands that had been worn by males in the aviary over the previous five months (GLMM: $F_{2,132}$ = 7.27, P = 0.001; Figure 2). In particular, both neutral-banded and red-banded males had a significantly higher song rate compared to the green-banded males after Tukey-Kramer adjustment for multiple testing (Green versus White: T_{132} = -2.94, adjusted P = 0.01; Green versus Red: T_{132} = -3.48, adjusted P = 0.002). On average, red males sang more than neutral males, although after Tukey-Kramer adjustment this difference was non-significant (White versus Red: T_{131} = -0.66, adjusted P = 0.79). Male song rate was also influenced by female response, with more song produced for

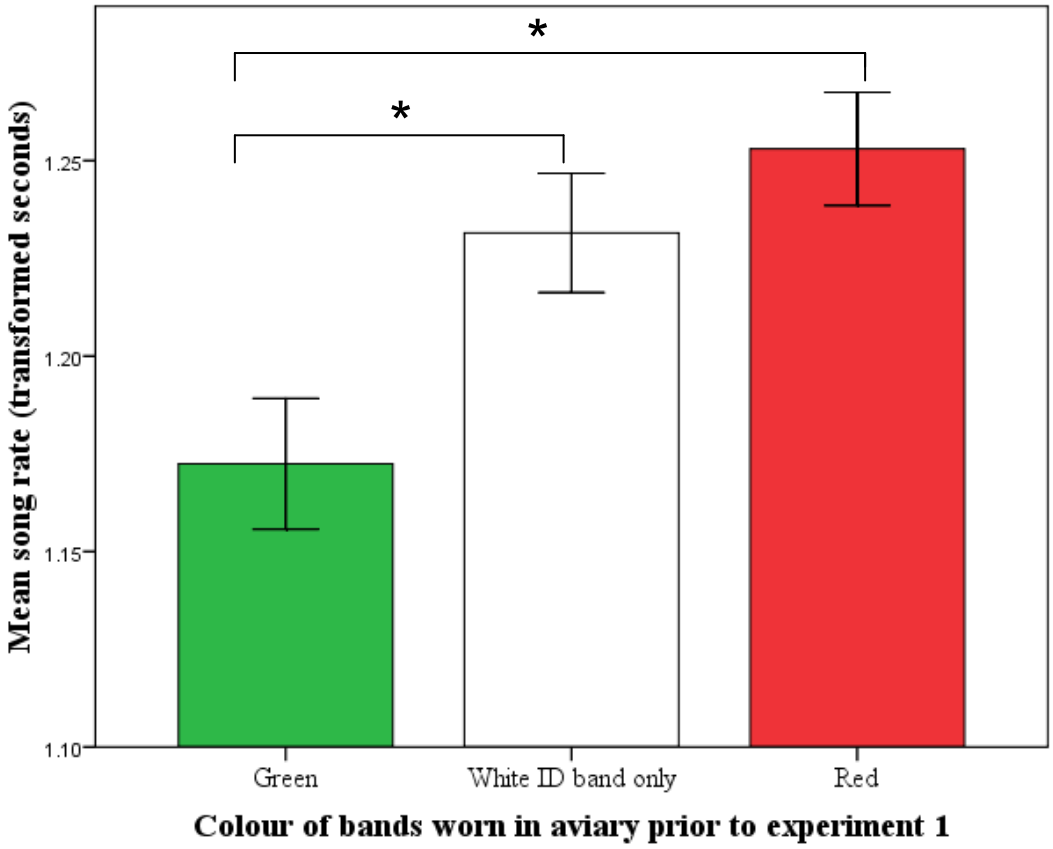


Figure 2: Mean male song rate (transformed seconds ± SE) for males that had worn green, neutral and red leg bands in the single sex aviary for five months prior to the mate choice trials.

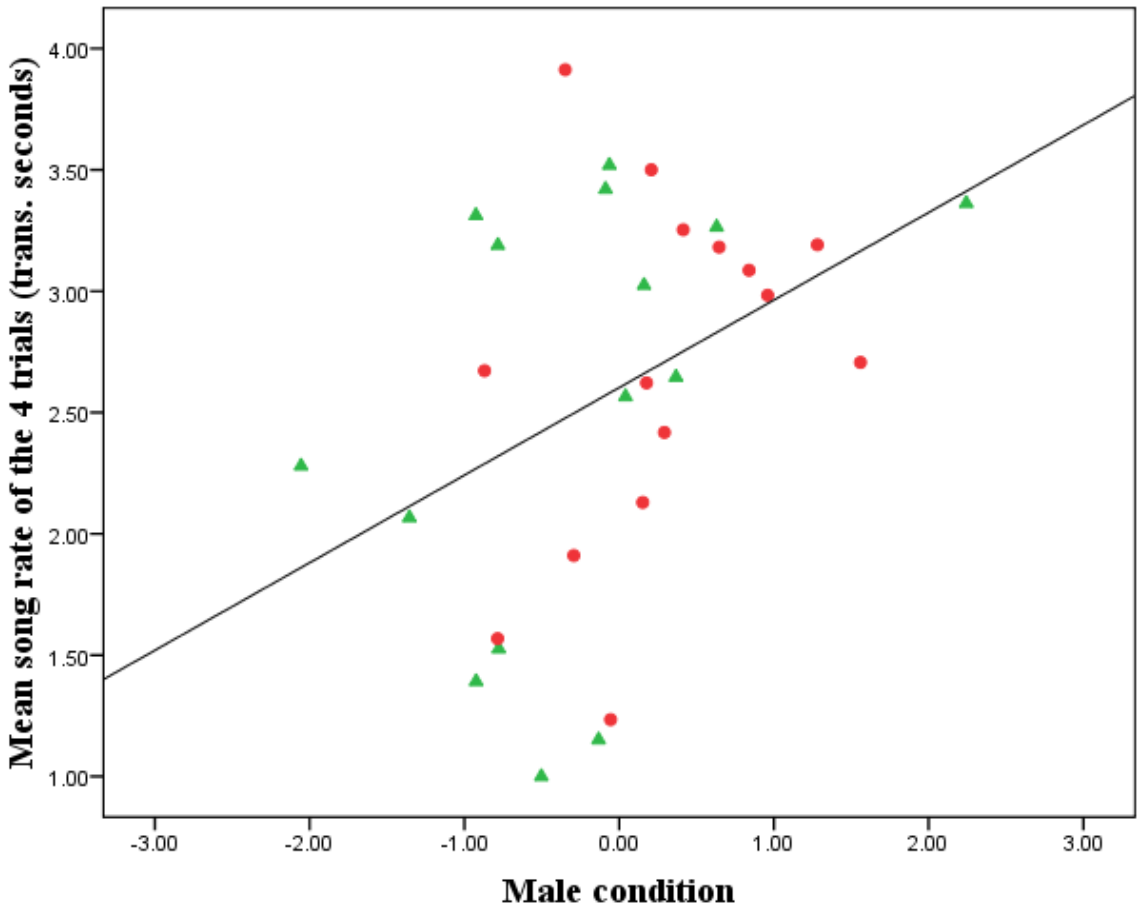


Figure 3: Mean song rate for the 4 trials conducted in experiment 2 (transformed seconds) plotted against male condition score (assigned from the residuals of mass:tarsus). The circles represent males that had been wearing red bands and triangles are males that had worn green bands in the aviary prior to experiment 2 mate choice trials. The fitted line is for all males combined.

females that showed a positive response during the trials compared to females that had a neutral/negative response to the male (GLMM: $F_{1,132} = 5.31$, $P = 0.02$). The colour of bands worn in the trials (i.e. the short-term band colour) and all other male and female variables measured were all non-significant and removed from the model (see appendix 10). Male song rate was highly repeatable between trials ($r = 0.701$, $F_{57, 116} = 8.04$, $P < 0.0001$).

In Experiment 2, in which no colour bands were worn in any trial, we found that male song rate was significantly influenced by male condition (GLMM: $F_{1,82} = 7.80$, $P = 0.007$; figure 3). In these trials, as shown in the morphology analysis, male condition varied between the red and green treatment groups, but direct influence of colour bands worn in the aviaries on song rate was non-significant and removed from the final model (see appendix 11a). Removing male condition from the model did not alter this result (see appendix 11b). Again, males sang more for females that expressed positive behaviours (GLMM: $F_{1,80} = 40.45$, $P < 0.0001$). Song rate repeatability in these trials was lower than for Experiment 1 (ANOVA: $R = 0.309$, $F_{29, 90} = 2.34$, $P = 0.001$).

Female response:

In Experiment 1, females showed a positive response to the colour bands worn by a male during the trial (GLMM: $F_{3,148} = 2.61$, $P = 0.0538$; Figure 4). Females demonstrated the highest preference for males wearing red and orange bands compared to green and neutral (white identity band only). Females also responded to male song rate, preferring males that sang more during trials (GLMM: $F_{1,148} = 14.54$, $P = 0.0002$). Trial number was also found to be influential in the model (GLMM: $F_{3,148} = 6.81$, $P = 0.0002$), with increasing positive responses with the number of trials (see appendix 12 for full model). In Experiment 2, in which females were presented with males wearing no colour bands, the only significant variable was male song rate (GLMMIX: $F_{1,89} = 20.89$, $P < 0.0001$). The band colour worn in the aviaries, male condition and trial number were non-significant and removed from the final model (see appendix 13).

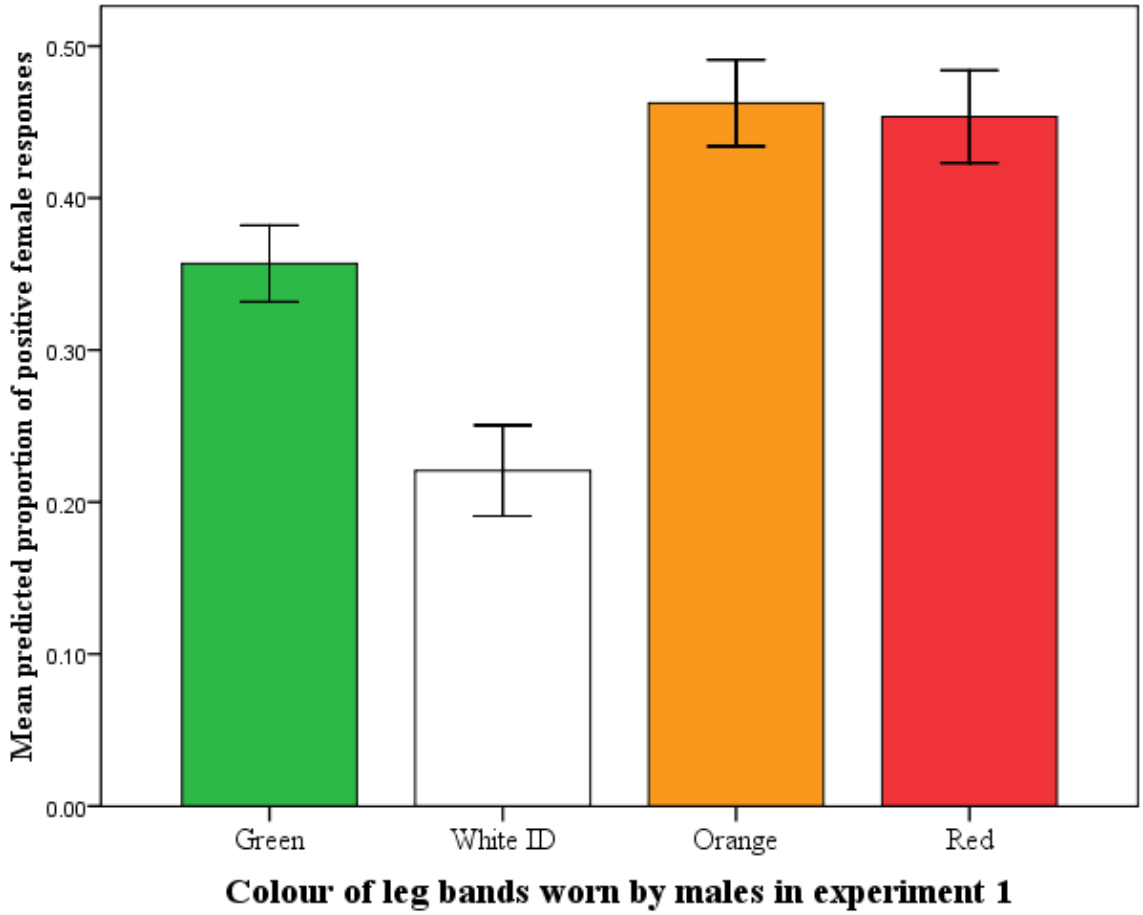


Figure 4: The mean proportion of positive female responses (± SE) to males dependent on colour band, as predicted by the final model of the no-choice trials conducted in experiment 1.

Discussion

We predicted that if band colours influenced male-male interactions, birds housed in a social environment for an extended period of time, would vary in condition and quality of condition-dependent sexually selected traits. After a period of 5 months in single sex aviaries, males that had been wearing red leg bands sang significantly more in mate choice trials compared to neutral and green banded males. During this experiment, male song rate was highly repeatable across four trials, suggesting an intrinsic difference between males. Song rate has repeatedly been shown to be a strong predictor of female preference in this species (Houtman 1992; Collins 1994; Collins et al. 1994; DeKogel and Prijs 1996; Balzer and Williams 1998; Forstmeier and Birkhead 2004; Rutstein et al. 2007), and we have confirmed this in both mate choice experiments presented here. The effect of band colour on song rate was not directly detected in the second mate choice experiment in which males wore no colour bands. However, following a significant amount of time under mixed-sex social conditions, males that had been wearing red leg bands were in significantly higher condition, which in turn was the strongest predictor of song rate. A direct influence of colour bands on song rate may not have been detected in these trials due to the reduced sample size (30 versus 47, because of natural mortality of these short-lived birds over the course of the 18-month experiment). Males also showed a lower repeatability of courtship song duration during these trials, suggesting a greater influence of female attractiveness/responses in the later trials. Both mate choice experiments found a relationship between female response and male courtship, which has also been demonstrated in earlier studies (Collins 1994; Rutstein et al. 2007). Although this coupling may lead to an increase in the association between trait and preference during the trials, when controlling for this association (in Experiment 1), we found that male song rate intrinsically varied between males from each treatment group. At this stage, all males had only been housed in single sex aviaries, suggesting that the male-male interactions had largely driven this variation in courtship rate.

When testing predictors of female responsiveness in the first set of mate choice trials (Experiment 1), we showed that females tended to prefer males wearing red bands to those wearing green bands. Although this result with wild caught birds was not quite significant (P=0.058), it is in line with previous studies on captive-bred

individuals (Burley et al. 1982; Burley 1988b; Hunt et al. 1997) and thus confirms that female preference for red-banded males compared to green-banded males is likely to be a genuine bias in female choice rather than a by-product of captivity (Burley 1988a). In both mate choice experiments, females responded more strongly to the amount of courtship song performed by males during trials, although as previously mentioned, song rate is likely to involve some feedback from female response to the male. We did not detect any direct female preference for males based on the aviary colour band treatments. However, both experiments showed that colour bands affected male song rate, and thus indirectly affect female mate choice. The additive effect of colour bands on both female preference and male-male interactions may explain the physical differences between males of the two treatment groups being housed in the mixed aviary. Birds had also been allowed to breed over this period of time, potentially a more physically demanding environment compared to the single-sex aviaries. This may explain why, following this period, the most pronounced effects of band colour were on male condition. How long this influence on male physiology and behaviour lasts after removal of the colour bands is unknown, but it is likely that once a trait such as condition has been altered it may continue to provide an advantage to males in social situations, thus maintaining their relative superiority.

These multiple and interacting effects of leg bands on female preference and male quality could aid in our understanding of discrepancies between previous studies. This is especially true when comparing studies that have been undertaken in an aviary situation, to ones investigating birds paired in individual breeding cages. Burley's (1985, 1986a, 1986b, 1988a, 1988b) classic studies and theories were all developed using breeding populations of birds housed in aviaries, and although the dual effects on female preference and male dominance were noted, the implications of their influence was not considered fully. The assumption that colour bands do not intrinsically affect the condition or quality of a male has generally led researchers to attribute variation in offspring quality solely to maternal effects. More recent studies that have investigated this question using single pairs are likely to provide more accurate data on the action and strength of these maternal effects (Gil et al. 1999; Rutstein et al. 2004, 2005; Gilbert et al. 2005, 2006; Williamson et al. 2006), as any additional influence of male-male interactions are controlled. It remains possible that the social feedback between the female response to a manipulated male and his courtship behaviour (Collins 1994; Rutstein et al. 2007; this study) may also provide a

mechanism that will influence variations in male quality, even within paired birds. It is unlikely that these effects would be as pronounced as those seen in this study on aviary housed birds, but it would be interesting to investigate further.

The addition of differently coloured bands to the legs of male zebra finches has been one of the most widely used and replicated experimental manipulations of 'attractiveness' in the field of behavioural ecology. Our results demonstrate that the manipulation does not just affect the appearance of an individual (as is claimed or assumed by most of the studies that have used the technique) but also key physiological and behavioural parameters, that may then feedback to attractiveness or appearance. The fact that wearing colour bands altered the intrinsic qualities of the males in our experiments certainly raises interesting questions about the exact nature of the mechanism that relates band colour to intrinsic changes in song rate and attractiveness. However perhaps one of the key questions that urgently needs to be addressed is what the relevance of red and green colour bands is to a female zebra finch. From the outset (Burley et al. 1982) it has always been assumed that females responded to the red signal of the red band because that was most like the red ornamental bill of her partner (it was never quite so clear why females responded negatively to green bands, anymore than blue, orange or yellow). Our results (and earlier results by Cuthill et al. 1997) raise further questions about what we manipulate when we add colour bands to a zebra finch. It is possible that the females pay less heed to the bands themselves but are in fact listening to an acoustic signal that becomes correlated with the leg bands (through social feedback and unknown physiological pathways), or perhaps the observed female responses of differentially allocating resources to offspring (e.g. Burley 1988a; Gil et al. 1999) are simply driven by a male's direct effects on a female's physiology. For example red-banded males become more dominant (Cuthill et al. 1997) and display at a higher rate (this study) and perhaps that simply triggers stress/hormonal responses in a female 'forced' to pair with such a male. In an important recent study, Bolund et al. (2009) argued that because it is difficult to understand what exactly is being manipulated with red/green colour bands they would approach the study of differential investment in the zebra finch by measuring reproductive success in small captive populations that were not colour banded. Intriguingly they found that females actually invested more (egg size and concentration of testosterone) in the eggs of the least attractive males. Their interpretation of this pattern of investment was that perhaps in this species, females

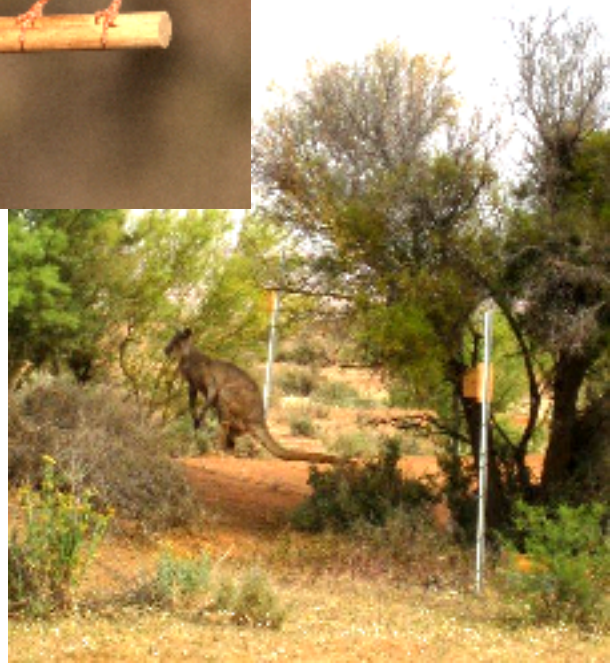
with the worst partners have to invest more as compensation for the poor quality of these males as social partners (Bolund et al. 2009).

There is no doubt that the red/green colour band manipulation in zebra finches has been a useful manipulation with respect to exposing some of the subtle investment strategies that female birds are capable of, however our findings not only provide a better understanding of the many influences that the addition of artificial traits can have on males, but also serve to highlight the importance of considering these effects when using coloured leg bands on captive populations of experimental birds. Further investigations using this manipulation will provide greater insight if we can better understand the pathways underlying the way that the bands affect intrinsic qualities and what, if anything they directly signal to females. Understanding those key questions will also serve to provide insight directly into the interplay between life history, physiology, social feedback, evolutionary fitness and the phenotypic plasticity of attractiveness.

Chapter 4

Differential female investment in response to male attractiveness: a study of zebra finches in the wild.

Emma Pariser, Jeff Graves, Lucy Gilbert, Lee Astheimer & Simon Griffith



I collected 90% of data; all hormone analysis was performed at the University of Wollongong by LA. I carried out 100 % of analysis and write-up, with comments and advice from LG and JAG.

Abstract

The Australian zebra finch is a classic avian model species. However the majority of work has focused on the domesticated variety, which has been bred in captivity with little input of wild genes for at least 40 years. There have been discrepancies in data between replicated experiments testing theoretical models, such as the differential allocation hypothesis, and it has been postulated that this may have resulted from variations in selective pressures shaping a domesticated strain that bears little resemblance to its wild counterpart. To investigate this further we conducted an experiment to test differential allocation in a wild population of birds, breeding in nest boxes on a cattle station in New South Wales, Australia. Male attractiveness was manipulated using coloured leg bands, a method repeatedly used in experiments on domesticated birds. It has been shown that both domesticated and wild females find males wearing red bands attractive and green bands unattractive. The experiment followed a within-female cross over design, in which all pairs laid two successive clutches, for the first clutch the male was un-manipulated and for the second clutch the male was assigned to one of the two treatment groups. We tested for variation in inter-clutch interval, egg size, clutch size, yolk testosterone concentration, offspring mass and offspring immune function. For breeding pairs that had not been experimentally manipulated we also tested whether these response variables correlated with any paternal phenotypic traits. We found that a female had a shorted inter-clutch interval if her partner had been given green leg bands. However, no other variables tested significantly differed between the two experimental treatment groups. Significant positive correlations between female investment and male phenotypic traits were revealed. Egg size was larger in clutches fathered by males in better condition and females laid eggs with higher yolk T when paired to males with brighter beaks and larger cheek patches. Interpretation of experimental data was restricted by a limited sample size but the correlational study suggests that wild zebra finches may positively invest resources in response to male attractiveness, as has been shown in studies using domesticated birds. Additional experiments using wild birds would be necessary to make these findings more conclusive.

Introduction

The study of evolutionary and behavioural biology often requires the use of model species that can easily be kept and bred in captivity. This is of particular importance when studying life-history decisions, where almost all aspects of an animal's environment can be controlled and manipulated in the laboratory. The Australian zebra finch (*Taeniopygia guttata*) is a classic avian model species. These birds are opportunistic breeders that will rapidly reproduce whenever conditions become favourable, and can reach sexual maturity within 100 days (Zann 1996). Consequently, under appropriate conditions in captivity, multiple generations can be bred and studied within a relatively short time period.

A ban on the export of all Australian wildlife in 1960, however, has meant that the majority of zebra finches used in recent evolutionary biology experiments have been bred in captivity without new genetic input for many generations (Immelmann 1962 cited in Zann 1996). This fact has led to growing speculation about possible differences between wild and domesticated zebra finches (Forstmeier et al. 2007; Rutstein et al. 2007). Domesticated birds are significantly larger than their wild counterparts and when their mate choice decisions were tested they were found to pair assortatively with other domesticated individuals (Rutstein et al. 2007). Further, wild birds were shown to have greater genetic variation than domesticated stocks (Forstmeier et al. 2007). Domesticated birds are exposed to novel and different selective pressures compared to wild birds and it is possible that this has shaped a captive strain that is no longer an accurate model of the species in its natural environment. It is important, therefore, to investigate whether findings from studies using domesticated birds can be confirmed in wild populations in Australia.

Zebra finches have been used extensively as a model organism to test adaptive maternal allocation hypotheses (Burley 1981; 1986a; 1986b; 1988a; Gil et al. 1999; Gilbert et al. 2005; Gilbert et al. 2006; Williamson et al. 2006; Bolund et al. 2009). However, there are discrepancies between many of these studies which could potentially be due to genetic differences between various test subject populations (Forstmeier et al. 2007). Understanding maternal allocation strategies and the influence of maternal resources on offspring fitness and survival is essential to our

ability to both interpret animal behaviour and to develop accurate evolutionary models (Mousseau & Fox 1998). The relevance of such models is contingent on how well they represent the dynamics of wild systems.

Models of life-history theory predict that mothers should alter their investment in a particular breeding attempt according to its perceived value (Williams 1966). Assuming that females trade off investment between their current and future reproductive events, the positive allocation hypothesis predicts that females will invest more resources when paired with an attractive mate, while investing less when paired with an unattractive mate (Burley 1988a; Sheldon 2000). Conversely, the compensation hypothesis predicts that females will invest more resources when paired with unattractive males as a means of compensating for (a) the predicted reduction in parental ability due to his inferior quality and (b) the predicted inferior genetic quality of offspring (Saino et al. 2002; Gowaty et al. 2007; Gowaty 2008). Both theories have found support in experimental tests using domesticated populations of zebra finches. Females have been shown to increase levels of yolk androgens and antioxidants (Gil et al. 1999; Williamson et al. 2006) and produce chicks with higher begging rates (Gilbert et al. 2006) for males manipulated to appear attractive. Conversely, another recent study has shown that females will lay larger eggs, supplemented with more carotenoids, for unattractive males (Bolund et al. 2009).

To fully understand and explain maternal allocation patterns in this species it is necessary to investigate these questions in wild populations. Deposition of many of the resources being tested in these experiments, such as yolk androgens, has never been measured in wild birds. By improving our knowledge of this species in the wild we may be able to put current data into a more accurate context as well as being better able to explain discrepancies between studies.

Thus the aim of this study was to investigate whether wild zebra finches vary their allocation of resources in response to male attractiveness in the same way as domesticated zebra finches. To address this question we quantified female allocation of mass and yolk testosterone (T) to eggs, as well as offspring growth and immune response, in relation to (a) manipulated attractiveness and (b) natural phenotypic variation of males in a wild breeding population.

a) Manipulated male attractiveness

Male attractiveness can be easily manipulated in this species as females have been found to show mate choice preferences for males wearing red leg bands and avoid males wearing green bands (Burley 1982; 1988b; Hunt et al. 1997), a finding recently confirmed in wild birds captured from the same study site (chapter 3). By using an identical method to manipulate male attractiveness and following a similar experimental design to published studies on domesticated birds (Gil et al. 1999; Gilbert et al. 2006; Rutstein et al. 2004a) this study will allow direct comparisons to be made. We predicted that if domestication has not influenced maternal investment behaviour we will observe within female variation in allocation of egg resources (size and yolk T concentration) to clutches following the addition of coloured leg bands to males.

b) Male phenotypic traits

In addition to the experimental manipulation, we also investigated whether female variation in resource allocation would correlate with any sexually selected male traits expressed by un-manipulated individuals breeding in the study site. Among the many mate choice experiments conducted using zebra finches, it has been shown that females have preferences for males based on body size, beak colour, cheek and chest patches (see review by Zann 1996; Forstmeier & Birkhead 2004;). Again, if domestication has had no effect of female investment in this species we would expect to find correlations between female investment and male size, condition or sexually selected traits.

We predicted allocation patterns to reflect either a positive investment or compensatory investment strategy, both of which have previously been demonstrated in studies using domesticated birds (Gil et al. 1999; Bolund et al. 2009).

Methods

Study site

Fieldwork was conducted at Fowler's Gap Arid Zone Research Station (31^{o} 05'S, 142 ^{o} 43'E) from August to December 2007. The site was generally characterised as semi-arid rangeland, consisting of a small group of trees (*Acacia spp*.) surrounding a small

dam within a large sheep enclosure. A total of 52 identical nest boxes were installed on steel stakes (1.5m high) placed close to trees and bushes (see photo on cover page of this chapter). Boxes were approximately evenly spaced (4m apart) throughout the site, numbered and monitored daily from the beginning of the study. The site contained a centrally placed walk-in ground trap that was permanently filled with commercially available mixed finch seed (Golden Cob, Sydney, Australia). This trap was used to capture birds periodically throughout the study. It also provided a constant food source that encouraged breeding and helped to control for one highly variable feature of the environment that can influence maternal investment in this species (Rutstein et al. 2004b). Focal birds could also be captured using hand made spring-traps which, when placed on a nest box, are triggered by entering birds trapping them within the box.

Captured birds

Once captured, all birds were measured for mass, tarsus length and fat deposition. This allowed two separate measures of condition; either a quantitative measure, residuals from the regression of mass on skeletal size (tarsus length), or a qualitative measure of the amount of fat stored in the furculum, ranging from 0 to 5, such that 0 indicates no fat and 5 is convex and overflowing (Helms & Drury 1960). Beak colour was objectively measured using a reflectance spectrometer (see below) and digital photographs taken of all males for measurement of cheek and chest patch sizes (ImageJ analysis). Patch sizes and beak colour measurements were all taken in triplicate and the mean value used. All birds were provided with a numbered aluminum leg ring and a unique combination of plastic coloured leg bands (A C Hughes, Middlesex, UK). These combinations comprised only colours that had been shown to be neutral in previous breeding/mate choice experiments in this species (Burley et al. 1982; Burley 1985). A uniquely coded Passive Identification Transponder (PIT) tag (Trovan, The Netherlands) was attached to one leg band, which was then used to identify and log individuals visiting at nests via an electronic decoder (Trovan LID-650, The Netherlands).

Colour measurement

Beaks were measured at the base of the upper mandible using a USB2000+ Miniature Fiber Optic spectrophotometer (Ocean Optics Inc., Dunedin, USA) and a xenon light

source (Ocean Optics Inc., Dunedin, USA) with a fibre-optic cable in a 90º/90º angle. Reflectance was visualized using the program Avasoft 7 (Avantes, Eerbeek, Netherlands). The mean of three consecutive measures was used for further analysis. Reflectance spectra were then split into four quantal cone caches representing the four cones used in avian vision, denoted VS (Very Short wavelength), S (Short), M (Medium) and L (Long) using the freeware program SPEC (Hadfield 2005). This methodology has been developed to account for the passerine visual system by including the spectral sensitivity of the four visual cones (Hart et al. 2000). The three measurements per region were averaged and cone caches transformed into three log contrasts with the L cone catch as the denominator (following methodology of Hadfield & Owens 2006). The three log contrasts were analysed using principal components analysis to derive the first Principal Component (PC1), which explained 91.9% (Eigenvector, $c_1 = 0.93$, $c_2 = 0.98$, $c_3 = 0.97$) of colour variation for males and 93% (Eigenvector, $c_1 = 0.94$, $c_2 = 0.99$, $c_3 = 0.97$) for females. High PC1 scores therefore represent more light being reflected at all wavelengths (brighter beak).

Monitoring breeding:

All nest boxes were checked daily for any sign of nest-building or breeding. Boxes were then observed to identify breeding pairs via leg band colour combinations. If necessary, the nest box could also be switched for an identical box containing a PIT-tag reader which could log the identity of all birds entering the box. Once parents were identified, each breeding event was assigned to either; (a) experimental manipulation of male attractiveness, or (b) the male phenotypic trait study. Not all breeding birds were assigned to the experimental treatment as simultaneous removal of all clutches in the breeding colony may have led to mass desertion. To avoid this, at least 30% of breeding attempts did not have eggs removed at any one time. Also, many birds were captured and measured for phenotypic traits but never re-captured for experimental manipulation (Figure 1), thus these breeding attempts were included in the correlational analysis (Figure 2).

a) Manipulated male attractiveness:

To experimentally test for variation in maternal allocation in response to male attractiveness we investigated within-female allocation of egg resources between two successive clutches, as well as between-female comparisons at the chick stage (Figure

1). Once a breeding attempt had started, parents were identified and eggs were removed and replaced with a dummy egg on the day they were laid. Removed eggs were measured for length and width (0.01mm) with egg volume calculated using the equation: volume = 0.519 x length x width^2 (Romanoff & Romanoff 1949), they were then stored for later hormone analysis. Egg mass could only be measured for eggs that were removed and weighed using electronic scales. Therefore, to analyse egg size differences between successive clutches (in which the second clutch was not removed) it was necessary to use volume (this correlated significantly with mass for eggs in which both measurements were taken, $F_{1,335}$= 1145.68, P<0.0001, R^2 = 0.775; appendix 14). Once a full clutch was complete (determined by no additional eggs being laid following two days) birds were captured using nest-traps. Often the capture of one bird would lead to abandonment of the nest, but if possible both birds were captured and then dummy eggs could be removed. When males were captured they were given either a red (attractive) or a green (unattractive) leg band on each leg. The assignment of males to each treatment was sequential throughout the field season, with the first male given green bands. As this manipulation meant that a unique colour combination could not be used, PIT-tags were used to identify males at the nest. When the manipulated pair was found re-nesting within the study site, all eggs were measured as per the first clutch and the second laid egg was removed (and replaced with a dummy) for hormone analysis. First clutch data from manipulated pairs that failed to re-nest within the study site was used in the analysis of natural male phenotypic variation

The purpose of removing the entire first clutch was twofold: First, it allowed for investigation of variation in testosterone (T) concentration across the laying order, which has not previously been documented for wild zebra finches. Second, the within female variation in yolk T allocation could be investigated by directly comparing the concentrations in the second laid eggs of two successive clutches. All removed eggs were placed on an artificial incubator for 72 hrs to allow embryo development, after which they were immediately placed in a -20^{o}C freezer awaiting testosterone analysis (these embryos were lost in transit to the lab, thus no genetic sexing could be done or included in the analysis presented in this chapter).

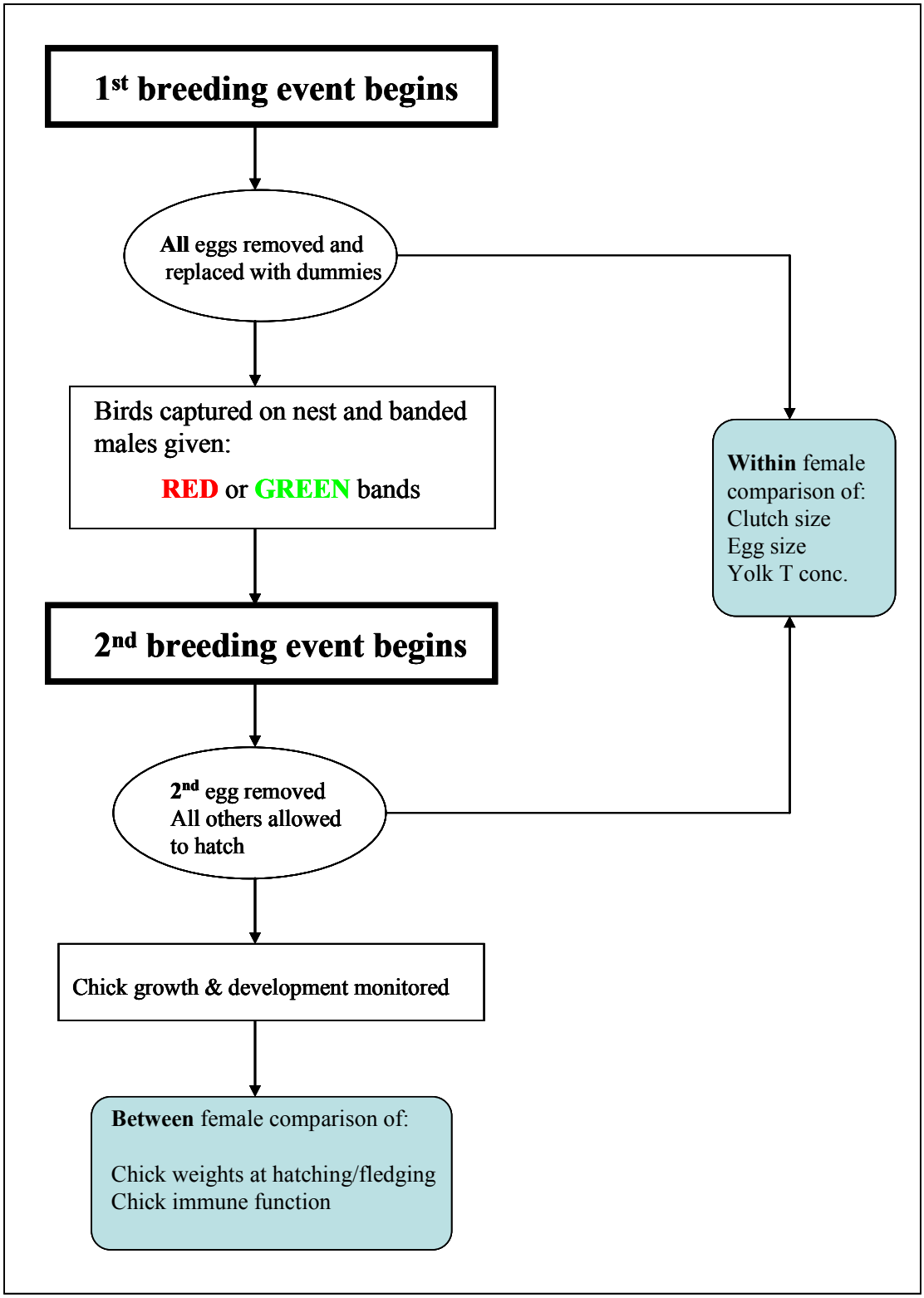


Figure 1: Flow diagram demonstrating protocol for experimental manipulation of male attractiveness and measuring female response.

Chick growth & immune response

For the second breeding attempt, offspring development was monitored daily. Brood size, hatching weight, weight at 10 days old (last weight that could be measured before risking force-fledging) and T-cell mediated immune response were recorded. Immune response was calculated by subcutaneously injecting 0.05 ml of buffered phytohaemagglutinin (PHA, 0.025 mg; Sigma Aldrich) solution into the wing web, following measurement using a thickness gauge (Mitutoyo Co, Japan). Wing web thickness was re-measured 24 h later in order to calculate the swelling response (no control injection in the other wing web was used, see Smits et al. 1999).

Testosterone Analysis

Whole yolks were removed and separated from the albumin using the different thawing rates between yolk and albumin. Yolks were weighed (0.01g) and homogenised using glass beads in 500 µl of commercial EIA assay buffer (Cayman Chemical, MI, USA). Each sample was spiked with 200 µl of labelled T to allow post-extraction recovery estimates. Samples were incubated overnight at 4^{o}C. T was then extracted twice for 90 minutes in 3 ml of a 30:70 (v:v) mixture of petroleum ether and diethyl ether. Ether extracts were removed after freezing the aqueous fraction in dry ice. The two extracts were combined, dried over nitrogen gas, reconstituted in 50µl ethanol, diluted 1:20n buffer and stored at -20 ^{o}C until assayed. T concentration was then measured using Cayman EIA assay kits (#582701). All yolks from the sample clutch/female were run on the same plate, in total samples were run on 11 plates (mean inter-plate variation = 25.35 %). T concentrations (pg/ml) were calculated by comparison with a standard curve. Mean extraction recovery for samples was 61.48% with a variance of 9.49%. This analysis was performed in Dr L. Astheimer's laboratory at the University of Wollongong.

b) Male phenotypic traits:

For clutches that were not assigned to the experimental treatment, we tested for correlations between natural male phenotypic traits and variation in maternal allocation of resources (egg size and T concentration). We also investigated any variation in other fitness measures such as nestling survival, development and

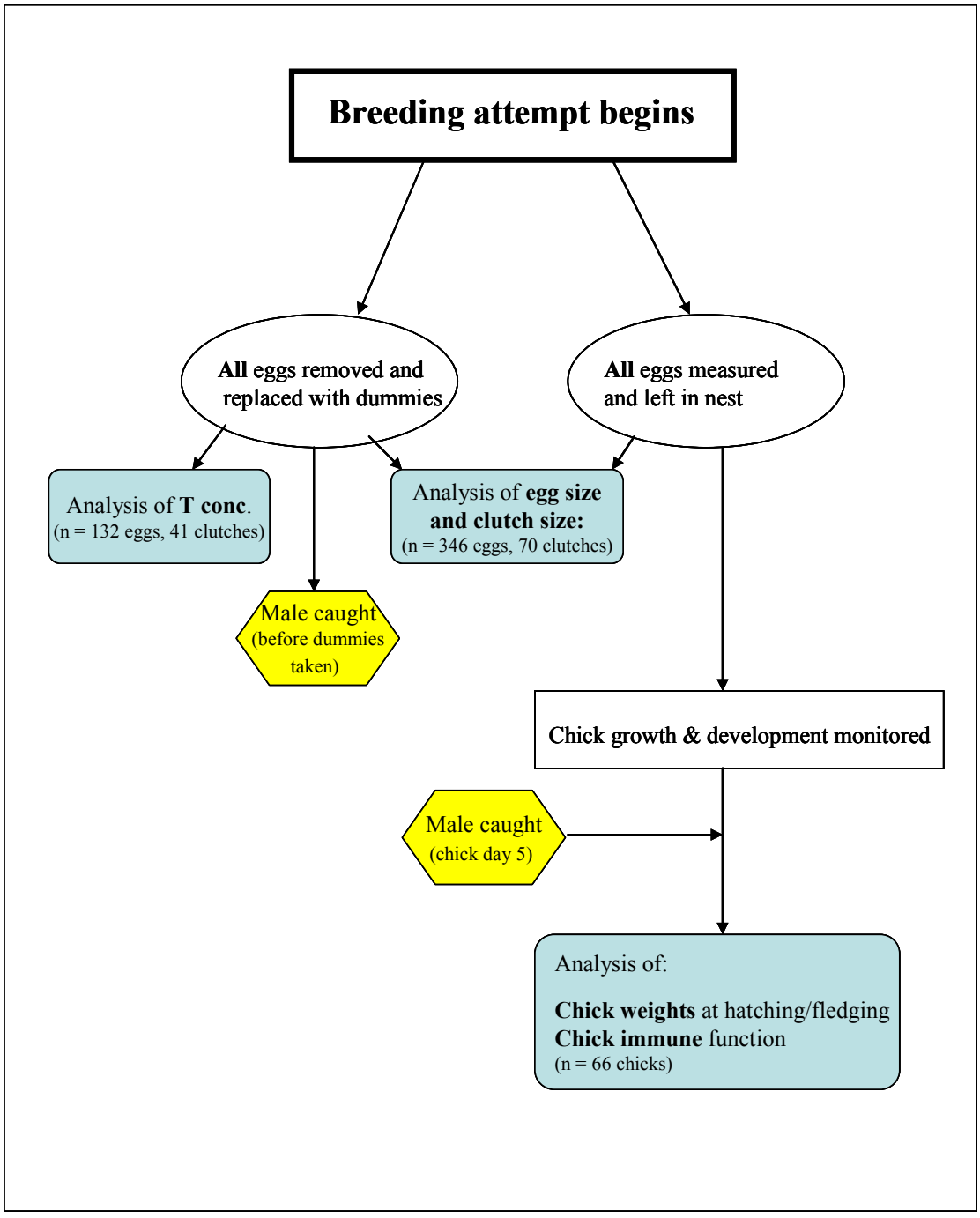


Figure 2: Flow diagram explaining the order of events for breeding attempts included in the analysis of correlations with male phenotypic traits.

immune response following the methods described above (Figure 2). These data included clutches that were removed for T analysis but not included in the experimental dataset due to (a) the male not being recaptured, or (b) the pair failing to re-nest within the study site. For inclusion in the correlational analysis, the male must have had full morphometric measurements taken.

Statistical Analysis:

a) Manipulated male attractiveness

For the within-female analysis, general linear mixed models (Proc MIXED) were performed in SAS version 9.1 (SAS Institute Inc, Cary, NC, USA) to analyse variation in egg volume and T concentration. Female identity was entered as a random factor in all models since several eggs are laid per female. Explanatory variables initially entered into GLMs were: male band colour, female condition or female fat score (in separate models), the laying order of eggs within the clutch, and clutch identity (first or second clutch laid). Two-way interactions between all variables and father's band colour were also initially entered and then a backwards stepwise procedure followed, removing non-significant variables ($p>0.05$) from the model starting with the least significant interactions. The Satterthwaite approximation was used to calculate denominator degrees of freedom (Littell et al. 2004). Identical models were used to investigate mean chick hatching mass, fledging mass and PHA response differences between treatment groups, except that, as these were data only from the second clutch, clutch order was not needed in the model. Similar models were used to test for differences in the number of hatchlings, fledglings and clutch size except for using Poisson distributed responses (PROC GLIMMIX).

b) Male phenotypic traits

When testing for correlations between female allocation and male phenotypic traits, identical models were used with the exception that male band colour was replaced with male beak colour, male cheek and chest patch sizes, male condition and male fat score as measures of quality/attractiveness. Male fat score and male condition significantly correlated with each other as did cheek and chest patch sizes. Therefore, two models were built for each response variable, one containing condition and cheek size, the other fat score and chest size. Again, female identity was entered as a random factor to account for multiple eggs laid per female and also multiple clutches per pair

of birds. Non-significant variables which were removed from the final models are not presented but all full models can are found in the appendices.

All other analyses were performed in SPSS version 16 (SPSS Inc, Chicago, Illinois, USA).

Results

a) Manipulated male attractiveness

There was no significant difference in female condition or fat score of pairs assigned to the two treatment groups (ANOVA; condition $F_{1,7} = 0.465$, $P = 0.517$, fat score $F_{1,7} = 1.722$, $P = 0.231$). A total of 21 males were caught and banded (11 red and 10 green) during incubation of dummy eggs replacing the first clutch. Of these, 7 red banded and 6 green banded males were recorded re-nesting within the study site, and thus were included in the experiment.

Within-female analysis:

Females paired to green banded males were found to have a shorter inter-clutch interval compared to females paired to red banded males (T-test: $t_{10.99} = 2.342$, $p = 0.039$; figure 3). Egg volume was not significantly influenced by the male attractiveness treatment (GLMM; $F_{1,4.956} = 0.32$, $P = 0.5938$). Females were found to lay larger eggs within their second clutch ($F_{1,62.06} = 19.26$, $P<0.0001$) but again this did not vary between treatment groups (breeding round*male band colour; $F_{1,62.06} = 0.03$, $P = 0.8702$; figure 4). Egg volume also varied across the laying order, with eggs increasing in size later in the clutch ($F_{6,62.12} = 9.96$, $P < 0.0001$; appendix 15). Female condition and fat score did not significantly influence egg volume and were removed from the final model (see appendix 16 for the full model).

Of the 13 experimental pairs, T could only be measured in the second egg of both clutches laid for 9 pairs (6 red banded and 3 green banded) due to eggs being lost during transport. Only the male attractiveness treatment entered into this model due to the very small sample size and it was not found to significantly influence yolk T concentration (GLMM: $F_{1,7} = 0.01$, $P = 0.92$).

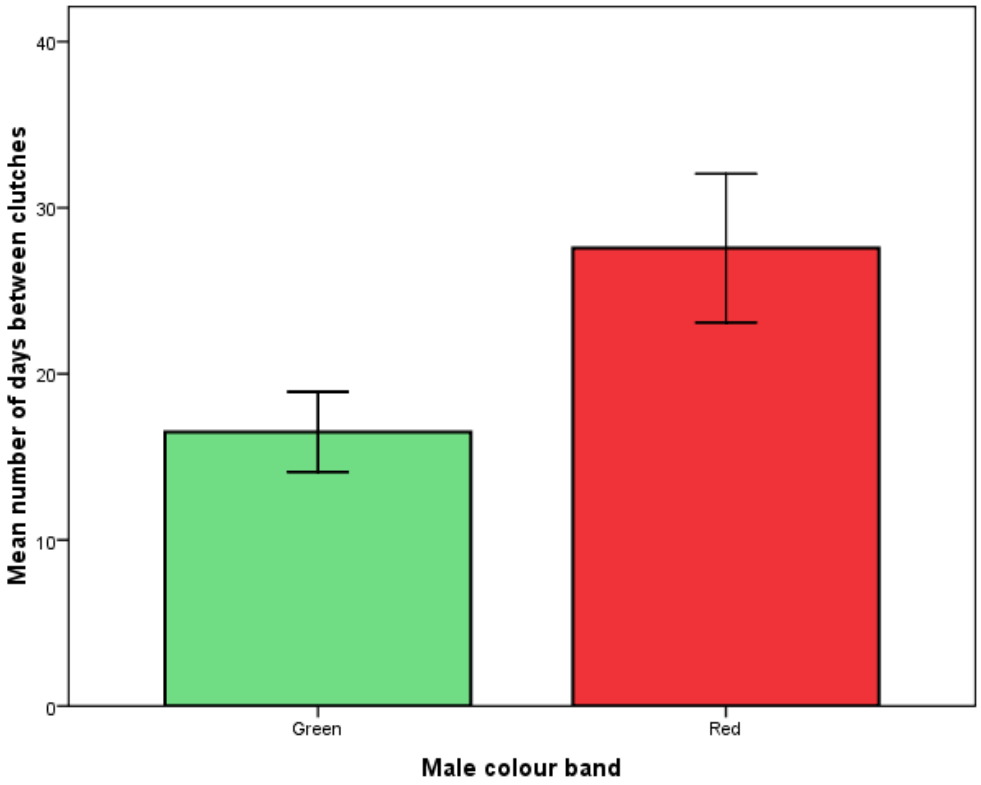


Figure 3: Mean number of days (±SE) between the first clutch laid for male prior to banding and the second clutch laid once males had been allocated either red or green colour bands.

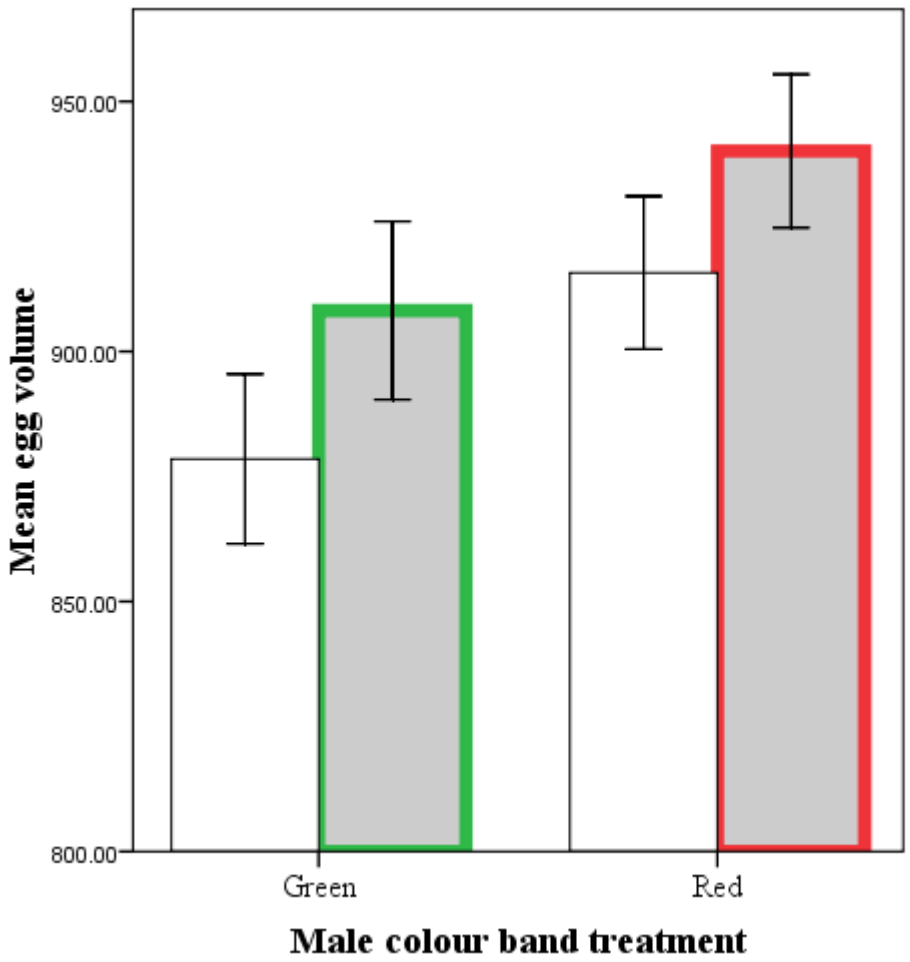


Figure 4: Mean volume in mm^3 (±SE) of eggs laid by females for their first and second clutches laid within the red banded and green banded male treatment groups (green n = 55 eggs, red n = 68 eggs). White bars represent the first clutch laid and grey bars represent the second clutch laid, after the addition of coloured leg bands.

Finally, there was no significant variation in clutch size between the first and second clutches laid by all females (GLMM with poisson error; $F_{1,1} = 0.30$, P = 0.6803) and this was not influenced by the colour band treatment that males were assigned to ($F_{1,1} = 0.19$, P = 0.7409). Female fat score and condition also had no significant effect on clutch size as shown in the full model (appendix 17).

Between female analysis

The total number of hatched and fledged chicks per brood did not vary significantly between red and green banded treatments (GLIMMIX with Poisson error; $F_{1,2.899} = 0.08$, P = 0.7931; $F_{1,1} = 0.00$, P = 0.9918; respectively). Female condition and fat score did not significantly influence either model (appendix 18).

There was also no evidence for a difference in average chick weight at hatching, weight at 10 days old or PHA response between clutches sired by red and green-banded males (ANOVA; $F_{1,4} = 0.01$, P = 0.923; $F_{1,6} = 0.016$, P = 0.904; $F_{1,4} = 0.048$, P = 0.837; respectively. Red banded father: *n* hatched chicks = 10, *n* fledged chicks = 8; Green banded father: *n* hatched chicks = 12, *n* fledged chicks = 12).

b) Male phenotypic traits:

In total, 346 eggs (70 clutches) were laid (and measured) by 39 pairs in which all males had been caught and measured. Pairs were tested for assortative pairing by size or condition measures, but no significant correlations were found (Pearson's correlations, all P> 0.05; appendix 30). As clutches contain multiple eggs per female and pairs laid multiple clutches over the breeding season, female identity was entered as a random factor in all models. Due to correlations between male traits (cheek and chest patch, male fat score and condition; beak colour and condition/fat score/chest patch; appendix 20), three separate models were built to test all response variables (a, b and c in appendices 21-30).

Females were found to lay larger eggs when paired to males with a higher fat score (GLMM; $F_{1,35.3} = 7.50$, P = 0.0096; figure 5). In addition, egg volume increased with laying order across the clutch (GLMM; $F_{1,279} = 47.4$, P < 0.0001). The number of successive clutches laid by the female was also found to influence egg volume (GLMM; $F_{3,292} = 3.32$, P = 0.0202) with females laying larger eggs as the number of

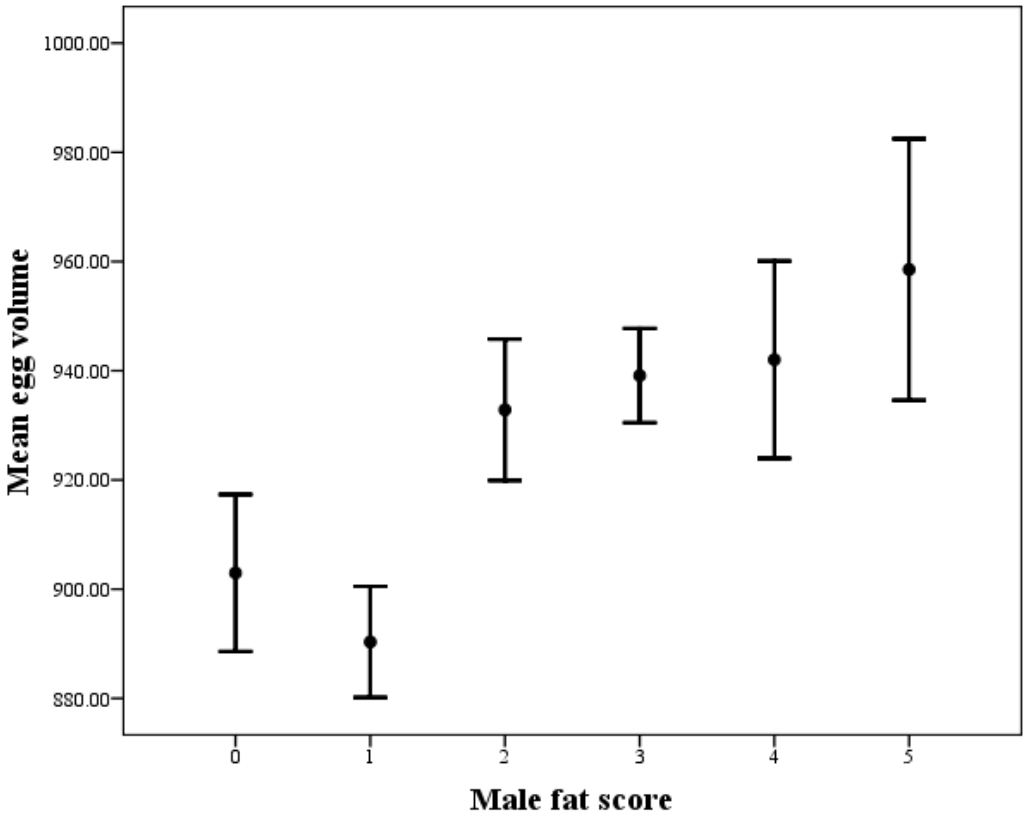
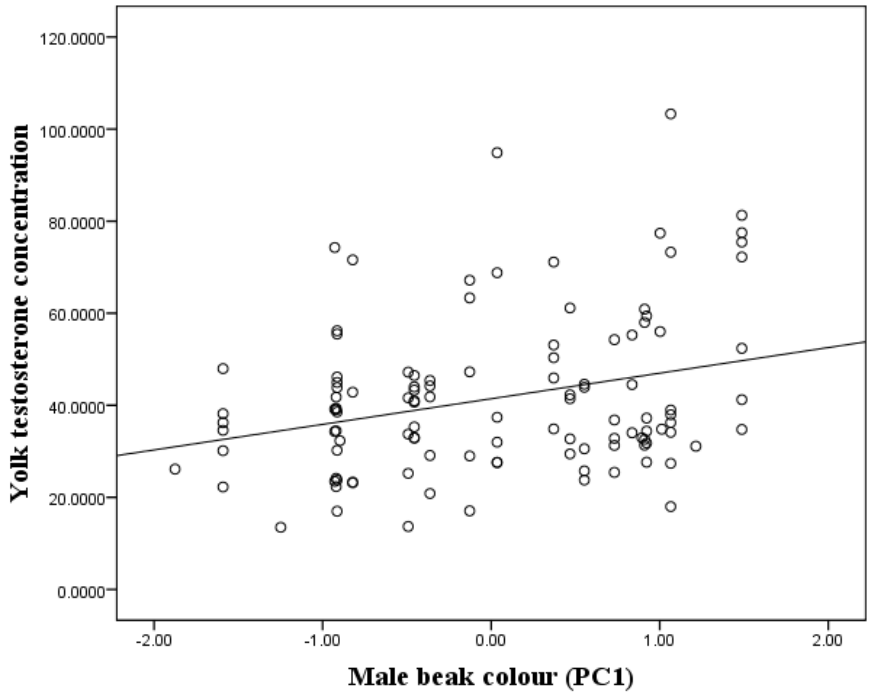


Figure 5: Mean egg volume (mm^3) ± SE, in response to male fat score.

a)



b)

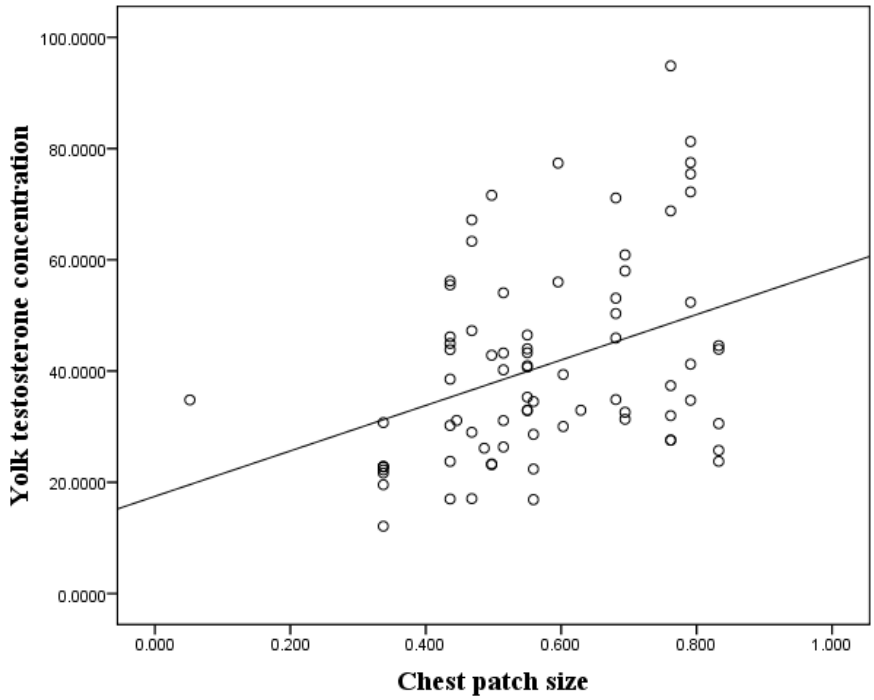


Figure 6: Yolk testosterone concentration (pg/mg) predicted by **(a)** male beak colour (first principle component of spectrometry data) and **(b)** male black chest patch size (cm^2).

clutches they had laid increased, however this result was strongly driven by one clutch of eggs laid in a fourth breeding attempt (see appendix 21). All other male phenotypic traits, along with female condition and fat score did not significantly correlate with egg size and were removed from the final models (see appendix 20).

Within this dataset, 132 eggs taken from 41 whole clutches were removed and analysed for yolk T concentration. Yolk T concentration was higher in eggs laid for males with brighter beaks (higher PC1 scores) (GLMM; $F_{1,110}$= 8.95, P = 0.0034) and larger black chest patches ($F_{1,14.8}$= 5.52, P = 0.0332; Figure 6). There was no evidence for any other male trait influencing yolk T concentration, so they were eliminated from all final models. Laying order also influenced yolk T concentration (GLMM; $F_{1,119}$= 8.49, P = 0.0043), with concentrations decreasing over the clutch (Appendix 23). Female fat score and condition did not influence yolk T concentration in any model and were consistently removed from all final models (see appendix 22 for full models).

There was no evidence that clutch size or the total number of hatched and fledged chicks could be predicted by any measured male traits (GLIMMIX with Poisson distribution; p>0.05 for all traits, appendices 24-26). We also found no evidence that chick mass at hatching was influenced by any male traits measured (appendix 27). However, males with brighter beaks had smaller chicks at aged 10 days ($F_{1,16.99}$= 6.07, P = 0.0247) but with greater immune responses ($F_{1,12}$= 9.23, P = 0.0103). Again, all other male phenotypic traits had no influence on the chick mass at day 10 or immune response models (see appendices 28 & 29). Female fat score and condition were entered as covariates in all models and were found to significantly influence both chick mass at 10 days and immune response. Females in higher condition had chicks that were heavier ($F_{1,23.6}$= 7.74, P = 0.0104) but with lower immune responses ($F_{1,12}$= 6.73, P = 0.0005; also for female fat score $F_{1,15}$= 15.30, P = 0.0014). Hatching order was only found to significantly influence immune response, with chicks hatching later in the clutch showing relatively reduced responses ($F_{4,34}$= 5.05, P = 0.0026).

Discussion

When male attractiveness was experimentally manipulated, colour band treatment was found to influence inter-clutch interval, with females laying their second clutch faster if paired with males that had been given green leg bands (unattractive treatment). However, we found no evidence for treatment effects on egg size, clutch size, yolk testosterone concentration, hatching success, fledging success, chick mass or immune function. The shorter laying interval by females paired to green banded males may align with the predictions of the compensatory hypothesis, which states that females will increase their investment in a reproductive event when paired to an unattractive male, in order to compensate for his reduced quality (Gowaty et al. 2007). However, this is assuming that a shorter laying interval represents an increased investment which may not be the case.

Contrastingly, female investment in response to naturally varying male phenotypic traits suggests that there is a positive association between male attractiveness and maternal allocation. Females laid larger eggs for males possessing larger fat reserves (a good condition indicator). Yolk T concentration was also significantly higher in eggs laid for males with brighter beaks and with larger chest patches – both potentially sexually selected traits in this species (Forstmeier & Birkhead 2004; Zann 1996). Both yolk T and egg size are solely maternally derived resources, however confirming this allocation pattern as differential investment is hampered by the possibility of assortative pairing of high quality females with high quality males. Although we found no evidence of assortative pairing based on size (tarsus length) or condition measures, it remains possible that birds could have paired using alternative cues. Offspring quality indices such as mass and immune function cannot be directly attributed to maternal variations in resource allocation as they can be equally influenced by variation in paternal input (either by direct or indirect/genetic means). Accordingly, we did find that both female condition indices and male phenotypic traits tended to correlate with chick size and immune function. Interestingly, more attractive males (brighter beaks) sired chicks with higher immune function but had a lower mass at 10 days old. The exact opposite was found for female condition; with mothers in better condition having chicks that were heavier at 10 days old but had reduced immune response. This suggests a potential trade-off

between size (or growth) and immune function of nestlings. Alternatively, offspring of mothers in a better condition may have lower baseline immune responses as they have acquired higher protection from maternal antibodies (Boulinier & Staszewski 2008). Finally, we found no evidence that any of measured male phenotypic traits correlated with the numbers of offspring hatched or fledged.

Positive or compensatory investment?

Our findings suggest that wild zebra finches differentially allocate resources in response to variation in male attractiveness. We attempted to investigate this in both an experimental manner, using direct manipulation of male attractiveness, and by correlating resource allocation by females with variation in trait expression in their mates. However, the potential contradictory nature of the direction of investment between the two methods means interpretation of the results is difficult. The experimental study is a more powerful means of investigating maternal allocation, as response variables were compared between two successive clutches laid by the same female, thereby controlling for between-female variation. However, due to the difficulties of using this design in a free-living population, only a very limited sample size could be achieved. Conversely, the investigation of male phenotypic traits had a much larger sample size but less experimental control of both within female variation and assortative pairing. Data from both studies were taken over the same period of time, within the same field site, so patterns of investment should be consistent. There is evidence for both positive investment (Gil et al. 1999; Rutstein et al. 2004a; Gilbert et al. 2006; Williamson et al. 2006) and compensatory investment (Bolund et al. 2009) in studies on domesticated birds and a recent model has suggested that female allocation strategy could be influenced by physical condition or age (Harris & Uller 2009). By using wild birds, the likelihood of potentially large individual differences in age and physical condition influencing results is increased relative to comparable laboratory studies. Therefore, although our findings suggest that wild females do differentially allocate resources, the data do not strongly support either the positive investment or compensatory investment hypotheses.

Wild versus domesticated birds

The central aim of this study was to corroborate captive studies reporting on differential investment in response to male attractiveness using a wild population,

ensuring that such findings were not an artefact of domestication. This study provides the first evidence that wild zebra finches adjust maternal resource allocation in response to male attractiveness. Our technique for manipulating male attractiveness was similar to that used by earlier researchers, in that we used colour bands to adjust perceived male quality and tested corresponding within female variation of resource allocation. In domesticated birds these types of experiments have shown that females paired with red banded males will; a) increase yolk T concentration (Gil et al 1999), b) increase relative concentrations of antioxidants across the laying order (Williamson et al. 2006), c) increase egg size (Rutstein et al. 2004a; Gilbert et al. 2006), d) increase the relative yolk concentrations of antioxidants to androgens (chapter 2) and e) produce offspring that have faster growth and development (Gilbert et al. 2006). Our data did not find any variation in female allocation of yolk T to clutches after their partner has been manipulated to appear unattractive. This would be in direct contradiction with both Gil et al. (1999) and Bolund et al. (2009) two studies that have demonstrated opposing allocation patterns in domesticated zebra finches. Our data are highly limited by the small sample sizes obtained and the environmental variations imposed by conducting such a study in the wild. Females were found to positively allocate both yolk T and egg size in response to certain sexually selected male traits, providing some evidence that wild birds are following similar allocation patterns to domesticated ones, but again, future work would benefit from more controlled investigation of these questions

In conclusion, in this study we have demonstrated differential maternal investment of egg size in response to variations in male attractiveness. This is the first investigation of these questions in free living, wild birds and, although hampered by the limitations of working with wild birds, the data presented are consistent with experiments following similar experimental designs on domesticated zebra finches.

Chapter 5

Maternal allocation in response to manipulated male attractiveness in socially breeding, wild caught zebra finches.

Emma Pariser, Simon Griffith, Amanda Gilby, Lee Astheimer, Lucy Gilbert, & Jeff Graves

I collected 80% of data; AG assisted with aviary work and all hormone analysis was performed at the University of Wollongong by LA. I carried out 100 % of analysis and write-up, with comments and advice from LG and JAG.

Abstract

The differential allocation hypothesis states that mothers should alter their investment in a specific breeding attempt in response to the attractiveness of her partner. Specifically, they should investment more in offspring that have been sired by an attractive male compared with what they would invest for an unattractive partner (referred to here as positive investment). The theory of compensatory investment predicts the exact opposite allocation pattern. Both hypotheses have found support in experiments using domesticated zebra finches, but neither has been tested on wild birds. To be able to draw direct comparisons with earlier work using domesticated zebra finches we used colour bands to manipulate male attractiveness and tested within-female variation in a cross-over design experiment. Wild birds were caught and experiments conducted in captivity, in two large outdoor aviaries. Males were given either red (attractive) or green (unattractive) leg bands and allowed to pair and breed. All eggs of the first clutch were removed and then males were recaptured and given the opposite band colour and the pair left to lay a second clutch. Only the second laid egg of this clutch was removed, with all remaining eggs allowed to hatch and offspring growth and development measured. The influence of the attractiveness treatment groups were tested on behaviour and maternal allocation at three stages: 1) Pre-laying: measuring assortative pairing and inter-clutch interval, 2) Egg laying: measuring egg mass, clutch size, yolk testosterone concentration, 3) Nestling stage: measuring chick weight and immune function. We found that females laid significantly heavier eggs when paired to males wearing red leg bands. A sex-specific interaction between offspring size and paternal attractiveness was also revealed, with the sons of red banded males being larger than daughters. No other response variables tested were found to be significantly influenced by the colour band treatments. Our data provide further support that female zebra finches invest more resources in a breeding attempt if paired to an attractive male (positive investment). This is the first experimental demonstration of differential maternal allocation in wild-caught zebra finches, validating earlier work on domesticated birds.

Introduction

'Maternal effects' is a term that broadly encompasses the various non-genetic ways that a mother influences her offspring (Mousseau & Fox 1998). This can range from deciding when or where to produce young, to some species expressing complex control over the specific size and sex of offspring (Trivers & Willard 1973). A mother is generally assumed to follow an allocation strategy that will optimise her inclusive fitness in the environment that she predicts her offspring will encounter. Therefore, she may vary resource allocation between reproductive events in response to factors such as current climate (Dentressangle et al. 2008) or season (Williamson et al. 2008), food availability (Rutstein et al. 2004b) or social conditions (Hargitai et al. 2009) to better equip her offspring, and ultimately enhance her own inclusive fitness. In iteroparous species, the attractiveness of a current reproductive partner can be considered an environmental factor that mothers may respond to. For example in birds, mothers have been found to lay larger eggs (Cunningham & Russell 2000), larger clutches (Petrie & Williams 1993; Balzer & Williams 1998; Uller et al 2005), and deposit more androgens, antioxidants and immunoglobulins in egg yolks (Gil et al. 1999; Saino et al. 2002b; Gil et al. 2004; Williamson et al. 2006) for attractive males. This positive investment in favour of attractive males is thought to be a result of mothers trading off resource allocation between current and future reproductive events, initially termed the differential allocation hypothesis (Burley 1988; Sheldon 2000). Contrastingly, females have been found to respond by increasing resources when paired to unattractive males. This has been observed with respect to egg size (Bolund et al. 2009), yolk androgens (Navara et al. 2006) and yolk carotenoid concentrations (Saino et al. 2002a). This is described as a compensatory strategy, and is proposed to explain increased maternal investment as a mechanism of mitigating costs related to the inferior genetic quality of the offspring's father (Gowaty 2008). Irrespective of which strategy is predicted to be acting, it is important to note that any variation in offspring fitness found to correlate with male attractiveness cannot be explained by inheritance of paternal genes alone, as variation in maternal investment in response to the male quality must be accounted for (Mousseau & Fox 1998).

In order to separate the potentially interacting effects of male genotype and phenotype from variation in maternal investment, it is necessary to experimentally

manipulate perceived male attractiveness. For this reason, the Australian zebra finch (*Taeniopygia guttata*) has become a model species for such studies, as male attractiveness can be easily manipulated using coloured leg bands (Burley et al. 1982; Hunt et al. 1997). Females have been shown to find the addition of red coloured leg bands attractive in males, and green bands unattractive. This manipulation has been effectively used in many studies to test maternal adjustment of egg resources, offspring growth, survival and sex-ratio (Burley 1981, 1986a, 1986b, 1988; Burley & Price 1991; Gil et al. 1999; Rutstein et al. 2004a; Rutstein et al. 2005; Gilbert et al. 2006). All of these studies, however, have used domesticated birds, captive bred in the USA or Europe for multiple generations, as test subjects. Domesticated zebra finches will breed readily if provided with appropriate conditions in captivity, even if separated into cages containing single breeding pairs (Gil et al. 1999; Gilbert et al. 2006; Rutstein et al. 2004a; Williamson et al. 2006). This is an important consideration in many experiments requiring control over between pair interactions, but this is a potentially impoverished environment for a gregarious and colonially breeding finch (Zann 1996).

In an earlier study we investigated maternal allocation in response to variation in male attractiveness in wild birds and found evidence that females will differentially allocate resources at laying in response to male attractiveness (chapter 4). However, the results of this study were not clear and limited sample sizes meant firm conclusions could not be drawn. Here we further test the questions posed in chapter 4, once again using wild birds, only this time within the controlled environment of an aviary. Similarly, coloured leg bands were employed to manipulate male attractiveness then female resource allocation was measured throughout two successive breeding attempts. To encourage breeding, birds were placed in large mixed groups in outdoor aviaries; which provided additional data on social interactions but also introduced potentially confounding factors: First, as birds were allowed to breed in mixed flocks, the possibility of extra-pair copulations and intraspecific brood parasitism (females laying fertile eggs in another nest) meant that molecular genetic testing was required to assign true parentage. Second, assortative pairing between attractive males and higher quality females may occur. This could be tested for and also accounted for by using a within-female cross over experimental design (direct comparisons between two successive clutches laid by each female, one clutch for each treatment group). Finally, our work on wild birds from the same

location has demonstrated the influence of colour bands on both female preference and male behaviour (chapter 2), thus we may expect an additive influence of the manipulation on birds in an aviary environment (i.e. if red bands increase both attractiveness and social dominance). Consequently, any difference in female resource allocation at the egg stage or later, would potentially be larger (or easier to detect) than in a more controlled, single pair breeding experiment.

Hypothesis testing

We tested response variables that had been shown to vary in relation to colour band manipulation in domesticated zebra finches: specifically, egg size (Rutstein et al 2004a; Gilbert et al. 2006), yolk T (Gil et al. 1999), and chick growth (Gilbert et al. 2006). In addition, high androgen concentrations have been shown to negatively influence immune function in the zebra finch (McGraw & Ardia 2007), thus if yolk T allocation varies, we expect it to reflect differences in offspring immune response.

In summary, as the positive investment hypothesis currently has the most empirical support in this species and aligns more closely with the reproductive decisions of an opportunistic breeder, we predicted that females would increase investment for attractive males in the following ways, measured at three reproductive stages:

1. ***Pre-egg laying***

If colour bands influence assortative pairing, females paired to red banded males should be larger, in better condition and have brighter beaks. These pairs should also pair faster and begin breeding earlier than green banded males.

2. ***Egg laying stage***

Females paired to attractive (red-banded) males should lay larger eggs with higher yolk testosterone concentration. As the aviary allows for extra-pair interactions, we predicted that females should have increased extra-pair paternity within their broods if paired to an unattractive male, and extra-pair sires should be relatively more attractive than the social mate.

3. Nestling stage

If females allocate more yolk resource (mass and T conc.) to offspring of attractive males, we predicted that they should grow faster, be larger at fledging, and have increased survivability. Conversely, they may show weaker immune responses.

Methods

Subjects:

Adult birds were caught in Sturt National Park, western New South Wales, Australia. Birds were then transported to Fowlers Gap Arid Zone Research Station (31^{o} 05'S, 142 ^{o} 43'E) and housed in single sex aviaries for 5 months. All birds were then transported to Macquarie University (Sydney), and in March 2008 were introduced into two large outdoor aviaries (10m x 8m x 2m). One aviary (A) housed 25 females and 25 males, the second aviary (B) contained 27 females and 27 males. All birds were weighed (0.1g) and measured for tarsus length (0.01mm), beak colour and fat score immediately before release into the aviaries. This allowed for both a quantitative and qualitative measure of condition: residuals of weight on skeletal size (tarsus), and the amount of fat stored in the furculum (0 = no fat, 5 = convex and overflowing, see Helms & Drury 1960).

Following capture, all birds were provided with an individually numbered white identity leg band. Prior to release into breeding aviaries females were also given a unique combination of plastic coloured leg bands (A C Hughes, Middlesex, UK). These combinations comprised only colours that had been shown to be neutral in previous breeding/mate choice experiments in this species (Burley et al. 1982; Burley 1985). Males were randomly assigned to either a attractive treatment (red bands on each leg) or unattractive treatment (green bands on each leg). A Passive Identification Transponder (PIT) tag (Trovan, The Netherlands) was attached to a coloured leg band on each individual. This, when scanned by an electronic reader (Trovan LID-650 decoder, The Netherlands), logged the time and identification of each bird entering or exiting a nest box.

Beak colour was measured at the base of the upper mandible using a USB2000+ Miniature Fiber Optic spectrophotometer (Ocean Optics Inc., Dunedin, USA) and a xenon light source (Ocean Optics Inc., Dunedin, USA) with a fibre-optic

cable in a 90º/90º angle. Reflectance was visualized using the program Avasoft 7 (Avantes, Eerbeek, the Netherlands). The mean of three sequential measures were used for further analysis. Reflectance spectra were then split into four quantal cone caches representing the four cones used in avian vision, denoted VS (Very Short wavelength), S (Short), M (Medium) and L (Long) (SPEC, Hadfield, 2005). This methodology has been developed to account for the passerine visual system by including the spectral sensitivity of the four visual cones (Hart et al. 2000). The three measurements per region were averaged and cone catches transformed into three log contrasts with the L cone cache as the denominator (Hadfield & Owens 2006). The three log contrasts were analysed using principal components analysis to derive the first Principal Component (PC1), which explained 96.3% (Eigenvector, $c_1 = 0.98$, $c_2 = 0.98$, $c_3 = 0.98$) of colour variation for males and 96.3% (Eigenvector, $c_1 = 0.99$, $c_2 = 0.98$, $c_3 = 0.97$) for females. Thus, high PC1 scores represent more light being reflected at all wavelengths (brighter beaks).

Housing:

Aviaries were outdoors and subject to daily fluctuation in natural light and temperature. This was approximately 12 hours of daylight and temperatures ranging between 18.1^{o}C and 25.3^{o}C (Australian Bureau of Meteorology). Both aviaries were of identical dimensions and contained 50 nest boxes evenly spaced ca. 1.5 m high and 0.5 m apart across two side walls and either side of a centrally placed supporting wall. Nest boxes were fully enclosed, with a circular entrance hole (30mm diameter), and accessible lid for monitoring breeding. Hay and feathers were provided throughout the breeding period for use as nesting material. All birds were provided with ad libitum mixed seed (Golden Cob, Sydney, Australia), cuttlefish, oystershell grit, spinach, sprouted green seed and water.

Experimental design:

To experimentally test for variation in maternal allocation in response to male attractiveness we investigated within-female allocation of egg resources between two successive clutches, as well as between-female differences at the chick stage (figure 1). Upon release into the aviaries all males were randomly assigned to a treatment group (aviary A = 12 red & 13 green; aviary B = 13 red & 14 green). All birds were freely able to select mates and begin breeding. Once a breeding attempt had begun,

parents were identified and eggs were removed as they were laid, weighed (0.01g) and replaced with a dummy egg. After a full clutch had been laid (no further eggs after 2 days), males were captured and given the opposite colour band treatment. Pairs were then allowed to re-nest. Once a second breeding attempt was identified, the eggs were weighed and labelled, but only the second laid egg removed and replaced with a dummy. Removal of the entire first clutch was necessary to investigate variation in yolk T concentration across the laying order, as this has not previously been documented in wild zebra finches. This data could then also be used to extrapolate T levels in the second clutch, using the second egg as a reference, to then correlate this variation with chick growth.

Egg incubation:

All removed eggs were individually labelled using a non-toxic permanent marker pen and placed on an artificial incubator (Brinsea, Somerset, UK) set at 37.5^{o}C for 72 hours. This provides enough time for the embryo to develop sufficiently for genetic analysis without significantly affecting yolk hormone levels (Gilbert et al. 2007). Once the incubation period was over, all eggs were stored at -20^{o}C to await further analysis. When frozen, eggs were split to remove embryos, which were placed in individual tubes and stored at -20^{o}C for later genetic sexing and parentage analysis. Whole yolks were then removed and separated from the albumin using the different thawing rates of yolk and albumin. All yolks were weighed (0.01g) using a digital balance, placed in labelled tubes and stored at -20^{o}C to await hormone analysis.

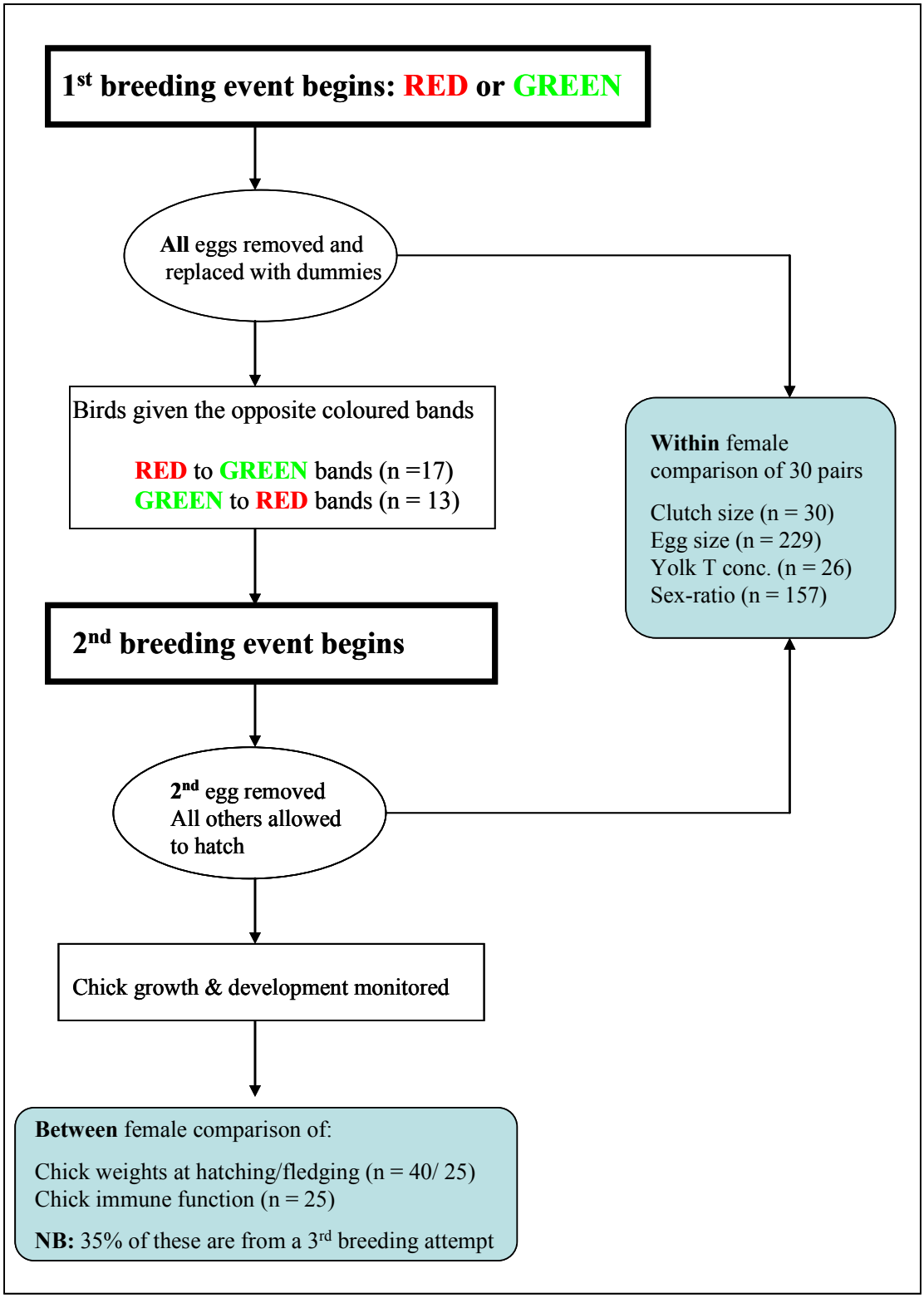


Figure 1: Flow diagram describing experimental design, analysis and sample sizes.

Testosterone analysis:

Frozen yolks were homogenised using glass beads in 500 µl of a commercially available EIA assay buffer (Cayman Chemical, MI, USA). Each sample was spiked with 200 µl of labelled T to allow post-extraction recovery estimates. Samples were incubated overnight at 4^{o}C. T was then extracted twice for 90 minutes in 3 ml of a 30:70 (v:v) mixture of petroleum ether and diethyl ether. Ether extracts were removed after freezing the aqueous fraction in dry ice. The two extracts were combined, dried over stream of nitrogen gas, reconstituted in 50µl ethanol, diluted 1:20n buffer and stored at -20^{o}C until assayed. T concentration was then measured using Cayman EIA assay kits (#582701). All yolks from the sample clutch/female were run on the same plate, in total samples were run on 11 plates (mean inter-plate variation = 25.35%). T concentrations (pg/ml) were calculated by comparison with a standard curve. Mean extraction recovery for samples was 61.48% with a variance of 9.49%.

Yolk T concentration was quantified for the single egg removed from the second clutch and was then used to calculate inter-clutch variability and estimate T concentrations in the remaining eggs via extrapolation (see appendix 32 for details).

Offspring development

All boxes were checked at least once daily and several times on days close to expected hatching, to ascertain hatching order and assign hatched chicks to eggs. If the hatch order or egg number was unknown for an individual, they were scored as the mean of the possible options (e.g. if two chicks had hatched from either egg number 3 or 4, both chicks were assigned 3.5). Chicks were weighed (0.01g) and tarsus length (0.01 mm) was measured pre-fledging on day 11. This was the last day on which nestlings could be measured and premature fledging avoided. To quantify immune function, T-cell mediated response was measured at 10 days old for all nestlings, via subcutaneous injection of 0.05 ml buffered phytohaemagglutinin (PHA, 0.025 mg; Sigma Aldrich) solution into the wing web, following measurement using a thickness gauge (Mitutoyo Co, Japan). Twenty four hours later wing web thickness was re-measured to calculate the swelling response (no control injection in the other wing web was used as this has been demonstrated to be unnecessary (Smits et al. 1999). After measurement of the swelling response all chicks had a blood samples taken (ca. 5µl) for genetic sexing and paternity analysis, and were given an individually

numbered, white plastic leg band. If a breeding attempt failed (i.e. all eggs failed to hatch within a clutch) the pair were left to lay a third clutch, with the male wearing the same band colour as for the second clutch. This was necessary due to poor weather conditions causing either nest desertions or poor embryo development. 35% of chicks analysed were from a third clutch because of this.

Genetic sexing and paternity analysis:

We extracted DNA from all embryos and blood samples using the Puregene extraction method (Gentra Systems). Part of the W-linked avian CHD gene in females and its Z-linked homologue found in both sexes was amplified using the polymerase chain reaction (PCR) using specific zebra finch primers ZF1 and ZF2 (Rutstein et al. 2004b). PCR's contained 10-50 ng of genomic DNA, 0.2 µl of 8mM dNTP, 0.2 µl each of 50 mM primers ZF1 and ZF2, 0.5 units of Taq polymerase (Bioline), 0.6 µl of 50 mM $MgCl_2$, 1 µl Bioline 10 x NH_4 reaction buffer which gave a total volume of 10 µl. The PCR products were separated on a 3% agarose gels at 50 V and visualised using ethidium bromide. Females had two bands at 350 and 384 bp and males had one at 350 bp.

We used five microsatellite loci to genotype all birds and offspring (Forstmeier et al. 2007) in one multiplex PCR reaction. Each reaction mix contained 10 µl QIAGEN® multiplex reaction mix, 3.8 µl RNase-free water and 0.2 µl of primer mix containing equimolar (0.2 µM) amounts of fluorescently labelled primers Tgu1, Tgu4, Tgu8, Tgu10 and Tgu12, and finally 1 µl of genomic DNA (15-25 ng). Amplification was by a step-down PCR protocol with an initial denaturing step of 15 minutes at 95°C followed by 10 cycles of 94°C for 30s, 64°C for 90s, 72°C for 90s. After this high stringency cycling the annealing temperature was reduced in a step-wise fashion from the initial 64°C to 60°C in the next 10 cycles, then to 56°C in 10 cycles and then to 50°C in the final 10 cycles. After these 40 cycles were complete there was a final extension period of 72°C for 10 minutes. This step-down PCR approach allowed co-amplification of primers with different annealing temperatures. All primers were fluorescently labelled using the WELLRED™ dye system (Beckman Coulter, CA, USA) which allowed alleles to be scored using a Beckman Coulter CEQ™ capillary sequencer and the CEQ™ 8000 Genetic Analysis System. Parentage was assigned using Cervus 3.0.3 (Marshall et al. 1998).

Statistical analysis:

For within-female analyses, general linear mixed models (PROC MIXED) were performed in SAS version 9.1 (SAS Institute Inc, Cary, NC, USA) to analyse variation in egg volume and T concentration. Female identity was entered as a random factor in all models since multiple eggs are laid per female. Explanatory variables initially entered were: male band colour, female condition or female fat score (in separate models), the laying order of eggs within a clutch, clutch identity (first or second clutch laid) and aviary identity. Two-way interactions between all variables and father's band colour were also initially entered and then a backwards stepwise procedure followed, removing non-significant variables ($p>0.05$) from the model starting with the least significant interactions. The Satterthwaite approximation was used to calculate denominator degrees of freedom (Littell et al, 2004). Identical models were used to investigate mean chick tarsus length and mass at 11 days old and PHA response differences between treatment groups, with the exception of clutch order as a factor (only second clutch analysed). All response variables were checked for normality and transformed if necessary.

Similar models were used to test for differences in the number of hatchlings, fledglings and clutch size, using Poisson distributed responses (PROC GLIMMIX). All other analyses were performed in SPSS version 16 (SPSS Inc, Chicago, Illinois, USA). Full and final models for all analyses performed can be found in the appendices.

Results

1. Pre-laying stage:

Out of the 52 males in two aviaries, 40 paired and completed at least one reproductive event with their partner (defining a breeding pair). Over the course of the experiment only 3 males changed partners, in these cases only the first pairing is considered for all of the following analyses.

Pairing:

Band colour did not influence the likelihood of pairing (GLIMMIX with binomial distribution; $F_{1,1} = 0.52$, $P = 0.6036$; appendix 33). There was also no difference in the

size, condition or beak colour of females that paired with red-banded or green-banded males (ANOVA; all $F_{1,38}$ <0.170, all P > 0.682; appendix 34). No correlations between female and male morphology, condition, or beak colouration were found (see appendix 35).

Latency to lay:

On average, pairs initiated their first clutch 23.48 days (± 14.86 s.d.) after introduction into the aviaries. Latency to lay did not differ significantly between red and green banded males (T-test; $t_{38} = 0.270$, $p = 0.789$; figure 2) or between aviaries (T-test; $t_{38} = 0.494$, $p = 0.624$).

2. Egg laying stage

30 breeding pairs laid two clutches, one for each treatment (17 for originally red-banded males and 13 for originally green-banded males). There were 229 eggs laid in 60 clutches (mean clutch size = 3.81, range = 2-6 eggs).

Genetic analysis:

157 offspring provided viable genetic material (either embryo or blood taken from chicks) that could be genetically analysed to ascertain sex and parentage. 99% of these samples were significantly assigned a mother first, then a father, using CERVUS. Two samples that could not be assigned parents were due to poor quality extracted DNA rather than problems with the microsatellites. 4 offspring (3%) were sired by an extra-pair male (3 in red treatment clutches and 1 in a green treatment clutch), and 6 offspring (4%) were a result of intraspecific brood parasitism (4 in green clutches and 2 in red). These levels of EPP/brood parasitism were too small to statistically test for any effect of treatment. The 6 parasitic eggs were not included in any further analyses of female resource allocation. The numbering of eggs laid within relevant clutches was also adjusted to reflect only the eggs that had been laid by the resident female.

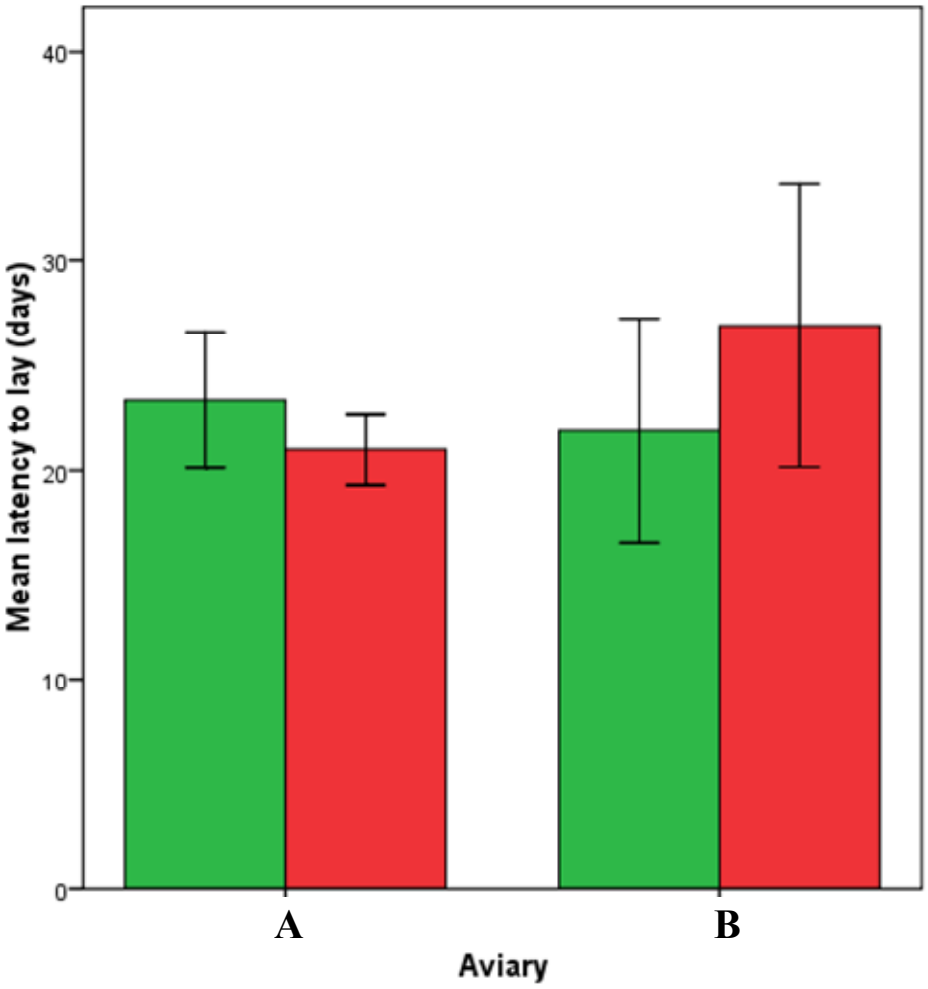


Figure 2: Mean latency to lay in days (± SE) the first egg of the first clutch for red and green banded males in each aviary.

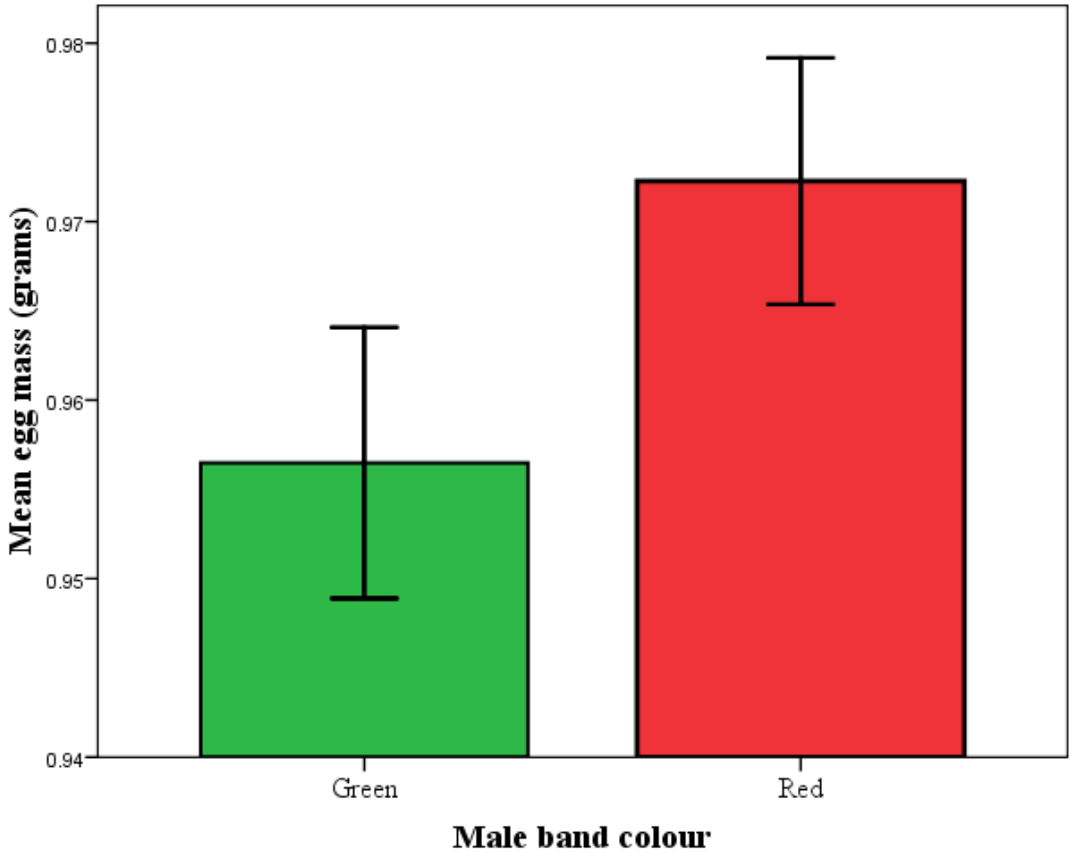


Figure 3: Mean mass in grams (± SE) of eggs laid by females paired to red and green banded males.

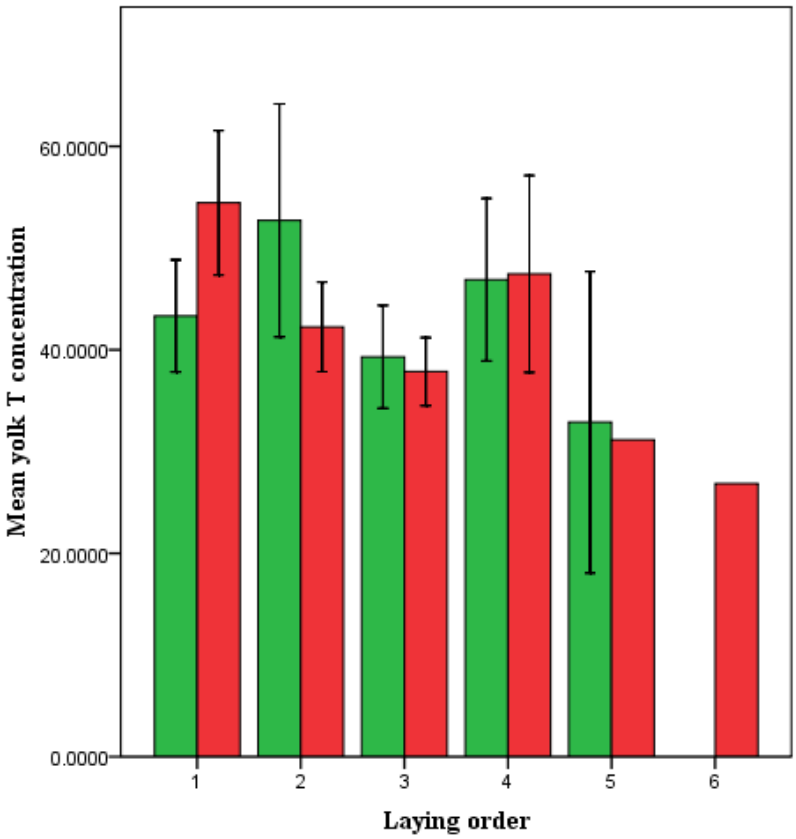


Figure 4: Mean yolk testosterone concentration (pg/ml) for first to sixth laid eggs in clutches laid by females paired to red and green banded males.

Egg mass:

Females laid larger eggs when paired to red banded males compared to green banded males (GLMM; $F_{1,189.7} = 4.91$, P = 0.0279; Figure 3). This result remained when the four known EPP offspring were removed from the dataset (GLMM; $F_{1,184.4} = 4.38$, P = 0.0378). Laying order, breeding round, aviary identity and female condition measures were non-significant and removed from the final model (appendix 36).

Yolk T concentration:

Due to egg breakages (2 samples) and problems with T extraction (2 samples), data from 26 breeding pairs could be analysed for variation in yolk T concentration (second egg from both clutches; *n* = 52). Male band colour was not found to significantly influence the yolk T concentration and was removed from the final model (GLMM; $F_{1,20.48} = 0.04$, P = 0.8356). No other variables entered into the model correlated with yolk T, apart from a trend for higher T concentration in eggs laid in aviary B ($F_{1,24.32} = 3.84$, P = 0.0616) and in the second clutch ($F_{1,24.06} = 3.63$, P = 0.0687; appendix 37).

Band colour did not influence yolk T concentration in first clutches (n = 24; GLIMMIX $F_{1,18.88} = 0.18$, P = 0.6727; figure 4). Within clutches, yolk T concentration significantly decreased over the laying order ($F_{1,66.44} = 13.66$, P = 0.0004) and eggs laid in aviary B were found to have higher T concentration than in aviary A ($F_{1,21.57} = 9.23$, P = 0.0061). Female condition and fat score were both non-significant predictors and were removed from the final model (appendix 38).

Primary sex-ratio:

There was no significant association between male band colour and the primary sex-ratio of clutches (GLIMMIX with binomial distribution; $F_{1,1} = 0.21$, P = 0.7246). No other covariates entered into the initial models were found to influence the primary sex-ratio and they were all sequentially removed (see appendix 39).

3. Nestling stage

Due to poor hatching success it became necessary to allow birds to lay a third clutch to increase sample size for the following analyses. Of the 30 females that laid two or more clutches, 20 clutches successfully hatched offspring (13 from the second clutch and 7 from the third clutch of eggs). The following analyses consider only those clutches in which at least one egg hatched.

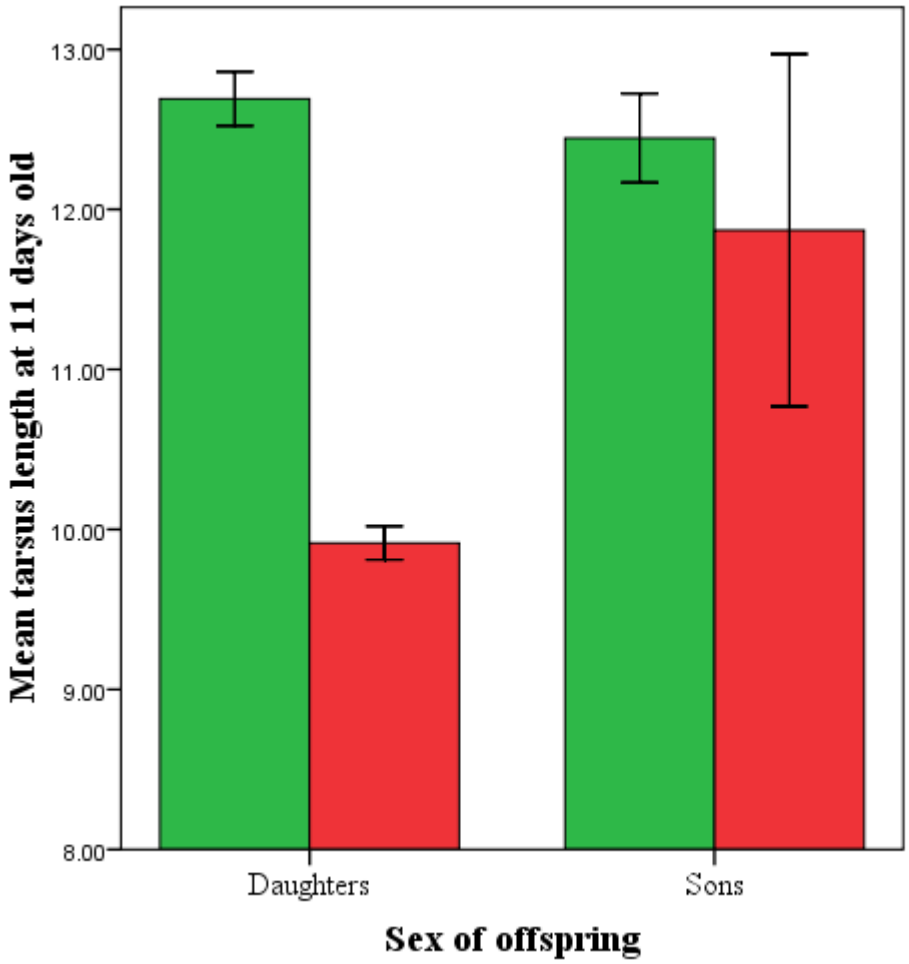


Figure 5: Mean tarsus length in mm (± SE) for daughters and sons at 11 days old, sired by red banded and green banded males.

Hatching success:

There was no significant difference in the proportion of eggs to hatch from clutches laid by females paired to red or green banded males (GLIMMIX with binomial distribution; $F_{1,1} = 0.74$, P = 0.5478; appendix 40).

Pre-fledging size:

There was a significant interaction between offspring sex and father's band colour (GLMM; $F_{1,11.83} = 7.71$, P = 0.0169; Figure 5). Daughters of red-banded males were smaller than sons (Tukey post-hoc tests; red-son: red-daughter $t_{12.04} = -2.92$, adj. p = 0.0549) but there was no difference between sons and daughters of green banded males (green-son: green-daughter $t_{11.18} = 0.48$, adj. p = 0.9620). Daughters fathered by red males were significantly smaller than daughters of green banded males (red-daughter: green-daughter $t_{16.23} = -3.60$, adj. p = 0.0169), but this difference was not found between sons (red-son: green-son $t_{16.23} = 0.96$, adj. p = 0.7721). T concentration, hatching order and female condition measures were all non-significant and removed from the final model (appendix 41).

No significant interaction of treatment and offspring sex was found when testing nestling mass differences at age 11 days (GLMM; Male band colour*chick sex $F_{1,7.8} = 4.20$, P = 0.0754). However, sons were generally heavier than daughters ($F_{1,8.804} = 8.27$, P = 0.0187). Estimated yolk T concentration significantly positively correlated with nestling weight at 11 days old ($F_{1,18.79} = 7.58$, P = 0.0127; appendix 43). Finally, the order in which nestlings hatched also correlated positively with weight at 11 days old ($F_{1,9.033} = 19.96$, P = 0.0015), with later hatched chicks being heavier. Both female condition measures and aviary were non-significant and removed from the final model (appendix 42).

PHA response:

Nestling immune response did not vary between chicks sired by red or green banded males (GLMM; $F_{1,6.733} = 0.03$, P = 0.8587). All other covariates entered into the model were also non-significant and sequentially removed (appendix 44).

Fledging success:

56.25 % of nestlings within clutches sired by red banded males fledged compared to 63.67 % of green banded male clutches. When tested, paternal band colour was not found to influence fledging success (GLIMMIX with binomial distribution; $F_{1,13.47} = 0.50$, P = 0.4900). All other covariates were sequentially removed from the final model (see appendix 45).

Discussion

We tested whether wild zebra finch females positively allocate resources towards offspring fathered by attractive males. Females significantly increased the mass of eggs laid for red banded (attractive) compared to green banded (unattractive) males. Offspring also showed sex-specific pre-fledging size differences dependent on their father's band colour treatment, with daughters of red banded males being smaller than sons. Further, daughters of green banded males were larger than those of red banded males. This result was not statistically significant when testing offspring mass, although the relationships were qualitatively consistent. Contrary to our predictions, manipulated male attractiveness had no effect on assortative pairing, latency to begin breeding, yolk T concentration, primary sex-ratio, rates of extra-pair paternity, nestling hatching success, immune response (PHA) or fledging success.

Egg mass:

The fact that wild females laid larger eggs for males manipulated to appear more attractive, supports data from studies on domesticated zebra finches (Rutstein et al. 2004a; Gilbert et al. 2006) as well as several other avian species (Cunningham & Russell 2000; Osorno et al. 2006; Loyau et al. 2007; Dentressangle et al. 2008; Bonato et al. 2009). It is well reported that heavier chicks hatch from larger eggs, which can have additive effects on chick growth and survival (Williams 1994; Christians 2002; Rutstein et al. 2004b). Interestingly, although egg size has been found to vary in response to mate attractiveness (Rutstein at al. 2004a; Gilbert et al. 2006) and diet quality (Rutstein et al. 2004b) in domesticated zebra finches, all of these studies found that differences increased across the laying sequence. In contrast, this study found no such within-clutch variation. This is unlikely to be due to differences between wild and domesticated birds, as similar data from free-living wild birds (chapter 4) also show a clear increase in egg size across the laying sequence. The present data potentially provide further support for a season effect on breeding behaviour (Williamson et al. 2008). This study was conducted during the Australian temperate autumn – winter season, not typical of the season or temperature conditions typically experienced by zebra finch populations breeding in the wild (Zann 1996).

When comparing studies using domesticated stock conducted in the same season, the lack of variation in egg size across clutches is generally consistent (Williamson et al. 2008). It is interesting to note that our data support the only existing study of seasonality in this species, and that zebra finches appear to be behaving similarly in the wild to in captivity (Williamson et al. 2008).

Yolk T concentration

Contrary to one previous study on domesticated zebra finches (Gil et al. 1999), we found that variation in allocation of yolk T could not be explained by the male attractiveness treatments. Our experiment differs from Gil et al.'s (1999) in both the source of the birds and also the sensitivity of the design. Both follow an identical cross-over design to detect within female responses to treatments, but Gil et al. analysed the androgen content in all yolks of both clutches. This significantly increases the sample size for investigating changes in T allocation both within and between clutches. By attempting to investigate within-female variation in allocation of some resources simultaneously with treatment effects on hatched offspring, we compromised on this aspect of the study. Thus, although we have no evidence for wild female zebra finches adjusting yolk T concentration between clutches in response to male attractiveness, the result could be attributed to low statistical power.

However, the use of this design allowed the investigation of correlations between offspring phenotype and estimated yolk T levels. As predicted, we found a positive association between chick size (mass) and the estimated T level of its egg. This corroborates direct injection experiments, which have demonstrated increased begging and growth rate in response to elevated yolk T levels in this species (von Engelhardt et al. 2006). Our data may be better explained by differences in chick competitive ability, as no association between pre-fledging skeletal size (tarsus length) was found.

Chick size

Skeletal size of offspring was found to be significantly affected by male band colour. Sons of red banded males were larger than daughters, which supports the theory of sex allocation (Trivers & Willard 1973; Weatherhead & Robertson 1979), but directly contradicts a previous study using this species (Gilbert et al. 2006). This study

differed from ours in the fact that offspring were cross-fostered, thus controlling for paternal influence.

In the present study, pre-fledging size differences cannot be solely attributed to maternal effects, as variation in paternal behaviour will have been influential. The demonstrated effects of colour bands on male dominance and behaviour (Cuthill et al. 1997; chapter 3) may go some way in explaining the differences between these studies. If red-banded males are socially dominant and/or in better physical condition (see chapter 3), this may affect feeding and nest defence behaviour, which could in turn affect offspring and/or maternal provisioning behaviour. Disseminating these inter-related influences and detecting underlying mechanisms would require further investigation. It is interesting to note, however, that our data corroborate an earlier study on aviary breeding birds in which offspring remained with their parents, where male-biased sex allocation was also demonstrated (Burley 1986a). Although studies controlling all aspects of reproduction are necessary to understand the potential mechanisms, it is also valuable to observe the ultimate outcome of these types of manipulation in a social context, as it more closely represents the species' wild ecology.

Assortative pairing

Surprisingly, we found no significant differences in female condition, size or beak colour between birds initially paired to attractive or unattractive males. Further, there were no significant correlations of morphological traits between individuals in breeding pairs. Unlike studies using domesticated birds (e.g. chapter 1), we also found no evidence for an influence of female condition or size on either egg size or yolk T concentration. If there were any undetected influences of assortative pairing or female condition on maternal deposition of the resources measured, they should have been controlled for by the experimental design and statistical methodology.

This chapter presents the first data showing that wild zebra finches positively adjust resources towards the offspring of attractive (red-banded) males, in line with earlier work demonstrating this effect in domesticated birds (Gil et al. 1999; Gilbert et al. 2006; Williamson et al. 2006). Earlier work (chapter 4) has suggested that wild females could adjust resources in relation to natural variation in male traits, but conclusive results could not be drawn. Using a more controlled, experimental

environment, these data support the predictions of the positive investment hypothesis (Burley 1988; Sheldon 2000), suggesting that domestication has not altered reproductive allocation strategies in this species. The factors causing some published data to align with predictions made by the compensatory investment hypothesis (Bolund et al. 2009) remain unclear, but it seems unlikely that population level differences are having major effects. A recent model showing female allocation strategies are flexible both individually and temporally (Harris & Uller 2009) may provide better explanations for inconsistencies in the published literature. These questions require further empirical testing, and future research should focus on teasing apart the various mechanisms involved in determining the maternal allocation strategies followed.

Chapter 6

General discussion, thesis summary and future directions

Emma Pariser, Lucy Gilbert & Jeff Graves

This thesis has investigated maternal allocation in response to male attractiveness in both wild and domesticated zebra finches (*Taeniopygia guttata*). Since the zebra finch is a model system for the investigation of avian life-history, sexual selection, signalling behaviour and maternal effects, the present findings are of importance to a broad array of behavioural ecologists. The central aims of the thesis were; a) To investigate how maternally derived resources (egg size, yolk testosterone and antioxidant concentrations) are allocated in response to males having artificially enhanced or reduced sexual attractiveness (chapter 2), b) To assess the behavioural responses of males and females to artificial manipulation of male attractiveness (chapter 3), c) To relate variation in female reproductive investment to natural variation in male secondary sexual traits (chapter 4), and d) To examine each of these phenomena for the first time using captive and free-living wild birds (chapters 4 & 5).

This concluding chapter seeks to synthesise what has been learned from the four studies and discuss how the findings relate to the current state of knowledge in this field. In addition, patterns of female resource allocation recorded in three different contexts (domesticated, captive wild and free-living wild) are directly compared, with the implications for inferring from domesticated studies to the wild context discussed.

Manipulating male attractiveness using colour bands

One of the main reasons that zebra finches have been used to test sexual selection theories over the past 20 years is that female preference can easily be manipulated by simply adding either red or green coloured leg bands (Burley et al. 1982; Hunt et al. 1997). Many naturally occurring male ornaments have been shown to influence female preference in this species (reviewed by Zann 1996; Forstmeier & Birkhead 2004), but the ability to adjust and reverse male attractiveness by changing leg bands has allowed for sensitive within-female tests of maternal allocation strategies and sex allocation (Gil et al. 1999; Rutstein et al. 2004a; Rutstein et al. 2005). Chapter 3 demonstrated that these colour bands can also influence social interactions in wild caught birds, in concurrence with an earlier study (Burley 1988b). Our study also confirmed female preferences for red over green bands in wild zebra finches, thereby validating the use of coloured leg bands in manipulating male attractiveness in chapters 4 & 5. However, we also found that the coloured bands influenced male song

rate and condition after long term exposure to the manipulation within aviary populations (chapter 3). This is an important consideration; although such effects do not alter predictions of female allocation at the egg laying stage in response to male attractiveness, they would need to be accounted for when investigating variation in offspring development. Males in better condition or that are more socially dominant may provision offspring more or defend a higher quality nestling environment (Wong & Candolin 2005), but this remains to be investigated in this species.

Chapter 3 demonstrated a clear feedback mechanism between female behavioural response and male courtship intensity, highlighting the fact that mate choice in this species is dynamic, something shown by an earlier study on domesticated birds (Collins 1994). As red bands have been shown to both increase male attractiveness (Burley et al.1982; Hunt et al. 1997) and male dominance (Cuthill et al. 1997), it is likely that traditional predictions of female egg resource allocation in response to male band colour would remain unchanged by such effects. Although our experiments showed no variation in male mortality between attractiveness treatment groups, it remains possible that the additive manipulation of social status may result in differential survival as demonstrated by Burley (1985), in which green banded males suffered significantly higher mortality than red. Burley ruled out competition between males as an explanation and instead suggested that red-banded males were less physically stressed during reproduction than the green banded males (supporting the later published differential allocation hypothesis (Burley 1988a). Although this may be true, we showed variations in a male secondary sexual trait (song rate) following housing in single sex aviaries, thus ruling out any influence of differential effects of reproduction. We can conclude, therefore, that male behaviour is affected by intra- and intersexual interaction and reaction to manipulated attractiveness. It would be interesting to investigate how long these changes in male quality persist after removal of the colour bands. It is possible that positive feedback via intrasexual interactions would maintain the social and/or physical advantage conferred to red-banded males, even after the bands were removed.

The findings of chapter 3 should be considered when designing aviary based studies. Experiments in which pairs are housed in individual breeding cages (for example chapter 2) would be recommended for investigating maternal effects in this species, in order to control the effects of the colour band manipulation. However, the decision to perform the experiment described in chapter 5 in aviaries was influenced

both by the need to provide newly captive birds with near-natural social conditions and by the fact that this design would allow for testing the effects of social interaction mediated by manipulating attractiveness.

Maternal allocation at laying

The primary focus of this thesis was to investigate maternal allocation strategies in response to manipulated male attractiveness. For such experiments there are currently two competing theories; predicting that females will either increase resource allocation when paired with an attractive male (positive investment), or invest more resources when paired with an unattractive male (compensatory investment). By manipulating male attractiveness using coloured leg bands we were able to experimentally test these theories using a sensitive within-female design.

Egg size:

Larger eggs tend to hatch larger chicks with a higher probability of survival (Williams 1994) and are more costly for mothers to produce (Monaghan & Nager 1997), suggesting that female control over allocation to egg size should be adaptive. We found that zebra finch females laid larger eggs when paired to attractive males; in response to both natural phenotypic variation (Chapter 4) and artificially manipulated attractiveness (Chapter 5). This was not the case in the manipulation of a wild population (Chapter 4) or the experiment using domesticated birds (Chapter 2). However, it is unlikely that these differences were due to intrinsic differences between wild and domesticated birds, since an earlier study has supported the hypothesis using the same domesticated population at St Andrews University (Rutstein et al. 2004a).

Overall, these results provide support for the positive investment hypothesis, and directly contradict a recent experiment on domesticated zebra finches which found females increased egg size for unattractive males (Bolund et al. 2009). This experiment differed from those presented in this thesis in that male attractiveness was not manipulated, but quantified using levels of extra-pair paternity. This methodological difference may go some way in explaining the contradictory results of these studies but further investigation would be necessary. Variability such as this

between studies exemplifies the need for replication using different populations or different methods to more accurately investigate evolutionary questions.

When comparing egg size data between the three studies (table 1) it is clear that domesticated birds lay larger eggs than wild. However, when factoring in female body mass, we found that females from all three groups (domesticated, captive wild and free-living wild) tended to lay eggs of proportionally the same size (approximately 7% of mass). We also found that egg size increased with laying order for both the free-living wild and domesticated birds, but not the captive-wild population. This may be associated with the relatively small clutches laid in this experiment (table 1) or a consequence of the colder temperatures (experiment ran in Australian autumn-winter), as seasonal differences in egg patterns across clutches has been shown in zebra finches (Williamson et al. 2008). Although domesticated birds are larger, relative investment by females into eggs is similar across origins and breeding conditions, as is the pattern of investment within clutches.

	Domesticated (n = 22 pairs, 97 eggs)	**Wild (n=37 pairs, 127 eggs)**	**Wild in captivity (n = 30 pairs, 108 eggs)**
Mean female mass (± SE)	16.43 g (±0.22)	12.94 g (± 0.08)	12.61 g (± 0.09)
Mean egg mass (± SE)	1.17 g (± 0.01)	0.96 g (± 0.01)	0.96 g (± 0.01)
Relative egg mass	7.12 %	7.42 %	7.61 %
Mean T conc. (± SE)	25.81 pg/mg (± 1.66)	40.08 pg/mg (± 2.28)	45.69 pg/mg (± 2.36)
Clutch size	4.71 (± 0.11)	5.05 (± 0.07)	3.76 (± 0.06)

Table 1: Descriptive statistics for the first clutch of eggs laid by birds included in the experiments performed in St Andrews (chapter 2), the wild (chapter 4) and in captivity using wild birds (chapter 5).

Yolk androgen concentration:

Female allocation of yolk T (or DHT) was not found to vary in response to manipulated male attractiveness in any of the experiments presented in this thesis. Positive allocation of yolk androgens in response to colour band-manipulated mate attractiveness has been demonstrated in two studies to date (Gil et al. 1999; von Engelhardt 2004) using zebra finches, and several times in other avian species (Gil et al. 2004; Gil et al. 2006; Loyau et al. 2007; Muller et al. 2008; Kingma et al. 2009). Conversely, many published studies have failed to find correlations between male attractiveness and maternal androgen allocation in various avian systems (Michl et al. 2004; Rutstein et al. 2004a; Gilbert et al. 2006; Dentressangle et al. 2008; Lopez-Rull & Gil 2009). This inconsistency among studies, between and within species, is difficult to explain and warrants further investigation. Egg yolk androgen concentration varies greatly between females (reviewed by Groothuis & von Engelhardt 2005), and within female tests are often required to accurately investigate female allocation strategies. However, this methodology has been employed for the majority of studies presented in this thesis (chapters 2, 4 & 5), suggesting that experimental design is not a likely explanation for the null results obtained. The one exception to this is chapter 4, where higher levels of yolk T were found in clutches fathered by males with brighter beaks and larger cheek patches, although this is purely correlational data. It is currently not understood how females transfer yolk hormones (reviewed by Groothius & Schwabl 2008) and this is essential for interpretation of data such as these. Specifically to understand whether yolk constituents vary due to a passive transfer from females, which have changed behaviourally or hormonally in response to the treatments they have been exposed to, or are part of an active process.

In chapter 2 we found a significant interaction between the amount of androgens (T and DHT) and antioxidants (total carotenoids and vitamin E combined) deposited in egg yolks. Females were found to vary the ratio of these two resources in eggs in response to male attractiveness and offspring sex. Females allocated less androgen relative to antioxidant for daughters when paired to unattractive males compared to attractive males. The interpretation of this strategy is problematic, as there is currently a lack of information regarding the importance of the relative concentrations of yolk constituents. With the growing sensitivity of methods allowing smaller amounts of yolk to be sampled, recent work has begun to investigate simultaneous deposition of these substances (Royle et al. 2001; Groothuis et al. 2006;

Navara et al. 2006; Safran et al. 2008). There is strong evidence that high levels of yolk androgen promote growth, development and survival (Schwabl 1993, 1996; Eising & Groothius 2003; Pilz et al. 2004; Navara et al. 2005; Tschirren et al. 2005; von Engelhardt et al. 2006; Muller et al. 2007), an effect supported by chapter 5, where we found a positive correlation between yolk androgen concentration and offspring mass at 11 days old. In addition, androgen levels have been shown to negatively influence immune function (Dufty et al. 2002; Groothius et al. 2005a; Navara et al. 2005), but we could not corroborate this effect by relating yolk T to offspring immune response. It is possible, however, that the interaction between these (or other yolk constituents such as immunoglobulin), have greater fitness consequences for offspring than each resource independently. Therefore, direct manipulation experiments are required to fully interpret the patterns of allocation and to differentiate between a costly or beneficial balance of yolk resources.

Comparing yolk T concentrations between the wild and domestic populations is difficult because of the different methodologies used for extraction and quantification. Accurate comparisons would not only require using the same technique, but for all samples to be analysed simultaneously to account for inter - assay variability. This was achieved for the free-living wild birds and the captive wild birds, thus these two groups could be compared. There was, however, no difference in yolk T concentrations between the populations (table 1). This is perhaps surprising, as variation in food availability has been shown to influence yolk androgen levels in some species (Dentressangle et al. 2008) and in zebra finches it has been found to influence only the pattern of allocation within a clutch (Sandell et al. 2007). We found no differences between domesticated, free-living wild and captive wild populations in the pattern of T allocation across clutches, with all females decreasing the concentration across the laying sequence (figure 1). This suggests that environmental differences between these did not significantly affect deposition patterns. The reduction in androgen concentration across the clutch has been repeatedly found in domesticated birds (Gil et al. 1999; Rutkowske & Cichon 2002; von Engelhardt 2004), thus it is reassuring to find this pattern is consistent in free-living wild birds.

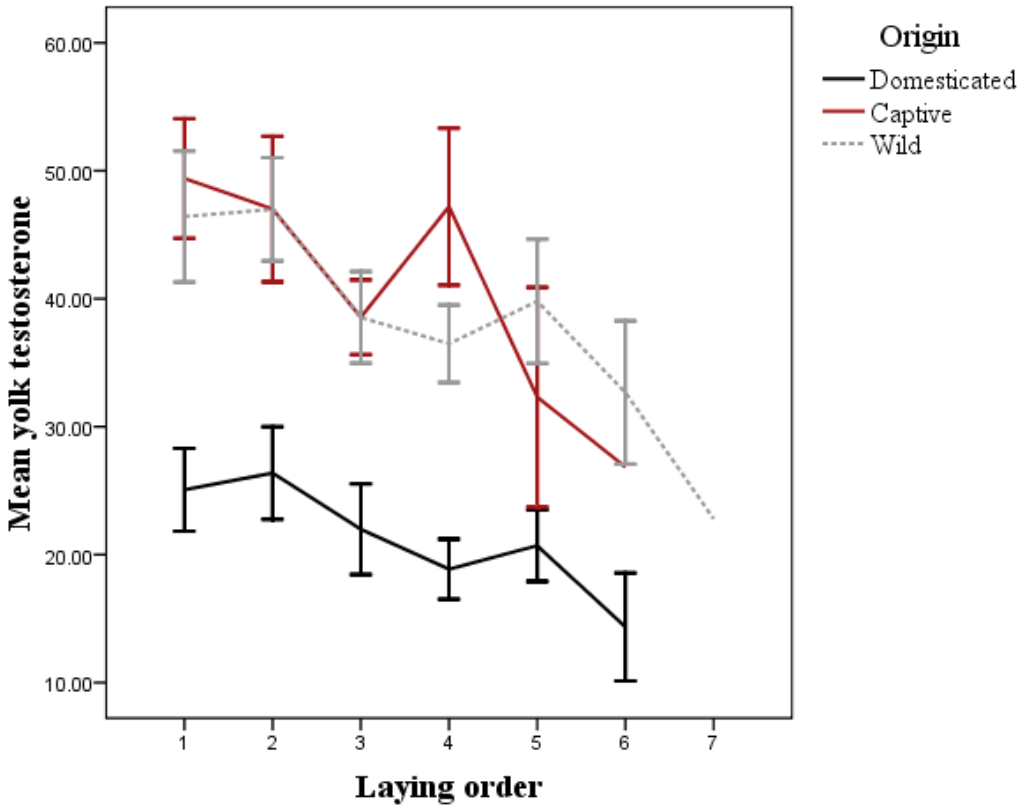


Figure 1: Mean yolk testosterone concentration in pg/mg (± SE) across the laying sequence for domesticated (chapter 2), free-living wild (chapter 4) and captive wild birds (chapter 5).

Are domesticated zebra finches a relevant model?

Domesticated zebra finches are significantly larger than their wild counterparts, approximately 30% larger, when we compare all individuals measured in experiments presented in this thesis. There is also evidence that males and females will assortatively pair when given a choice between a wild or domesticated sexual partner (Rutstein et al. 2007). Domesticated birds show reduced genetic variability compared to wild and there is significant genetic differentiation between domesticated populations around the world (Forstmeier et al. 2007). One aim of our study was to compare domesticated to wild birds, both between experiments and with earlier work following identical experimental designs.

We have confirmed that coloured leg bands influence social interactions among wild zebra finches (chapter 3) and are an effective means of manipulating male attractiveness in wild birds. This thesis also demonstrated that wild females will invest more egg resources (specifically egg size) for attractive (red banded) males. This result concurs with earlier studies that have demonstrated positive investment in domesticated birds (Burley 1988a; Gil et al. 1999; Rutstein et al. 2004a; Gilbert et al. 2006; Williamson et al. 2006). Patterns of allocation across the laying sequence of both egg size and yolk androgen content were also similar for wild and domesticated birds in our study. No accurate comparisons of total concentrations of androgen could be made due to variations in technique (Groothuis & von Engelhardt 2005), but it was found that domesticated birds housed at St Andrews University laid larger eggs than their wild counterparts. However, when accounting for relative body size, there are is no increase in the proportional size of eggs laid. Wild zebra finches were found to follow the same allocation patterns and life-history strategies as domesticated birds; this concurs with a recent experiment investigating chick growth and development (Tschirren et al. 2009). Therefore, we must conclude that zebra finches remain a relevant model for evolutionary ecologists. Any variation between our wild studies and the current literature appear to be no greater than the present inconsistencies between published studies on domesticated zebra finches.

Future directions

The data presented in this thesis suggest some interesting future avenues of research, since many of the studies present more questions than answers.

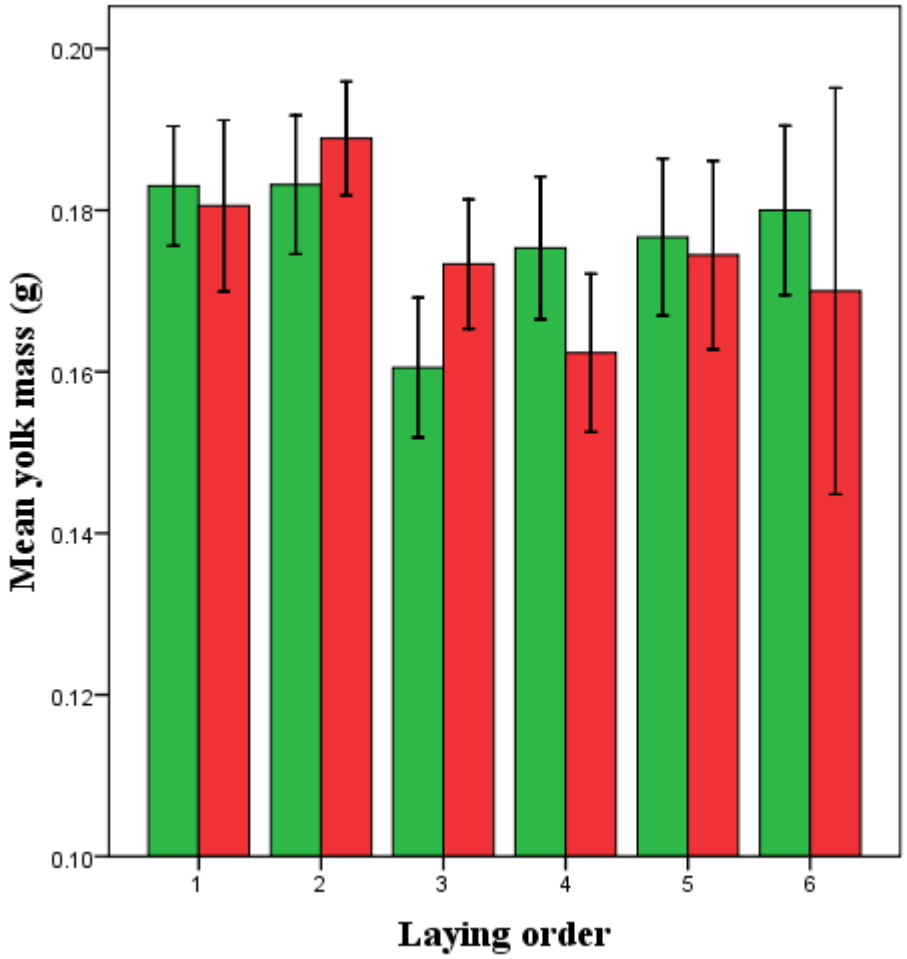
1. The experiment investigating yolk androgen and antioxidant ratio (chapter 2) definitely requires further investigation. There are currently few data available on either the balance of egg yolk resources or the importance of this balance to offspring fitness. Many researchers are directly manipulating yolk hormone levels using direct injection methods, but it would be useful to broaden this approach to investigate varying more than one yolk constituent. Androgens and antioxidants are obvious candidates; both are known to affect offspring fitness parameters (Blount et al. 2003; von Engelhardt et al. 2006), and have opposing influences on immune function (Dufty et al. 2002). The predictions made in chapter 2 were based on current theory, but solid empirical data is needed to accurately interpret the data.

2. A consistent theme throughout this thesis is the current conflict between two opposing theories of female differential allocation. Traditionally, differential allocation referred to a theory first proposed by Nancy Burley in 1988. This has been termed the positive investment hypothesis throughout this thesis in order to make a distinction from the more recent compensatory investment hypothesis (Gowaty 2008). The two theories predict diametrically opposed patterns of female allocation in response to male quality/attractiveness. Confusingly, support for both theories has been demonstrated in studies on the same species, in both the zebra finch (Gil et al. 1999; Bolund et al. 2009) and mallard ducks (*Anas platyrhynchos*) (Cunningham & Russell 2000; Bluhm & Gowaty 2004). With the growing empirical evidence for both investment strategies, it is important to investigate mechanisms that may explain these differences. A recent mathematical model (Harris & Uller 2009) suggests that female allocation strategy may be influenced by environmental conditions such as food availability, and female condition or age. The data presented in chapter 2 showed that sex allocation patterns varied dependent on male attractiveness and female condition. However, experiments in which multiple different environmental effects are independently varied would be useful to explore this idea further. It may be that current allocation models are too simplistic, and incorporating a measure of female state would yield more accurate predictions.

Appendices

- Appendices 1 – 9 Chapter 2
- Appendices 10 – 13 Chapter 3
- Appendices 14 – 31 Chapter 4
- Appendices 32 – 44 Chapter 5

Appendix 1: Yolk mass (chapter 2)



Mean yolk mass in grams (± SE) across the laying sequence for clutches laid for attractive (red banded) and unattractive males (green banded) from the experiment presented in chapter 1. There was no significant change in the weight of yolks inside successive eggs laid across clutches ($F_{172} = 2.301$, $p = 0.131$).

Appendix 2: T model (chapter 2)

Variable	df	Full model		Minimal model	
		F	p	F	p
Male band colour (MBC)	1, 35.6	0.06	0.8144		
Female condition	1, 32.8	2.23	0.1446		
Female fat score	1, 41.6	5.69	**0.0217**	7.84	**0.0075**
Laying order	5, 121	10.31	**<0.0001**	10.75	**<0.0001**
Breeding round	1, 33.1	0.01	0.9180		
Embryo sex	2, 130	3.40	**0.0365**	3.57	**0.0307**
MBC*female condition	1, 32.8	0.01	0.9232		
MBC*female fat score	1, 41.6	0.00	0.9892		
MBC* laying order	5,121	1.47	0.2036		
MBC*breeding round	1, 33.1	0.11	0.7380		
MBC*embryo sex	2,130	1.08	0.3416		

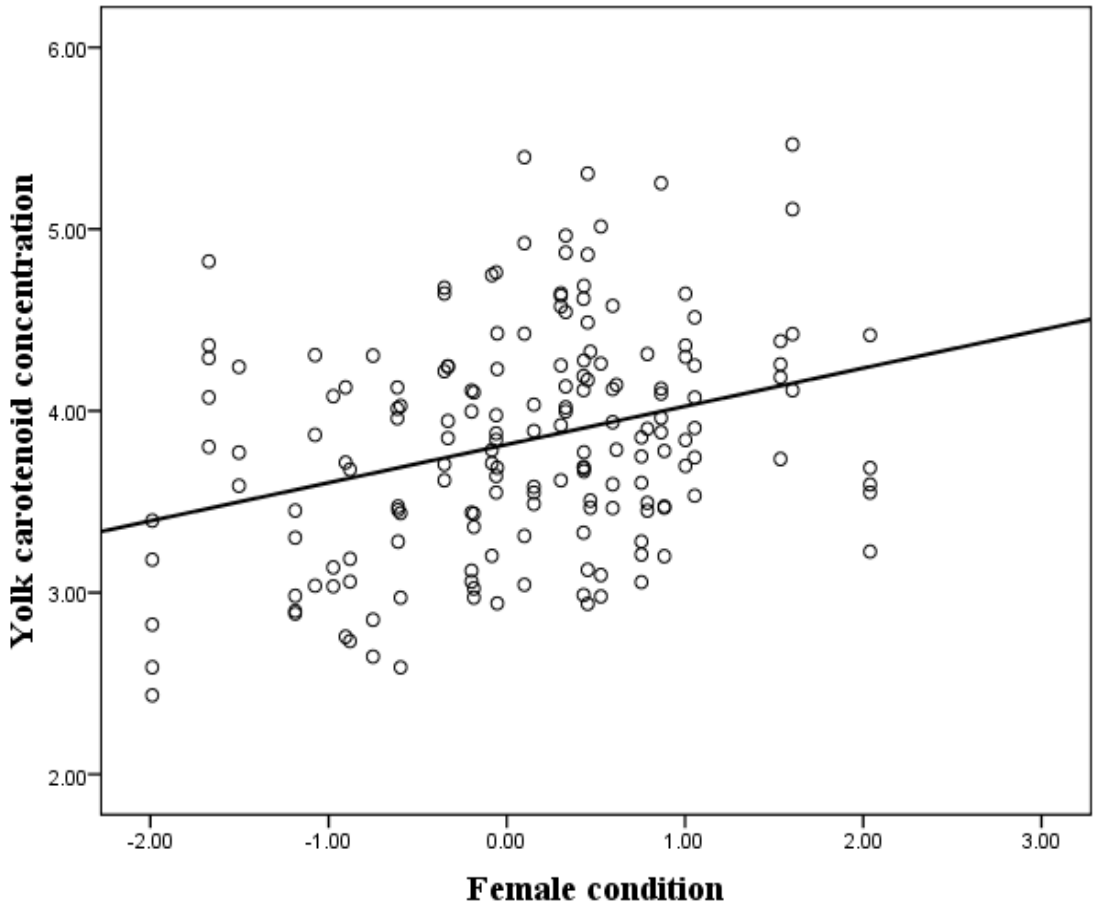
General linear mixed model analysis of T concentration within egg yolk to investigate the influence of male colour band treatment. Female identity was entered as a random effect, female condition and fat score were entered into two separate models and significant variables are shown in bold.

Appendix 3: DHT model (chapter 2)

Variable	df	Full model		Minimal model	
		F	p	F	p
Male band colour (MBC)	1, 38.4	0.03	0.8592	0.06	0.8057
Female condition	1, 32.9	3.18	0.0838		
Female fat score	1, 38.3	6.79	**0.0130**	8.29	**0.0064**
Laying order	5, 122	2.73	**0.0227**	2.86	**0.0175**
Breeding round	1, 33.3	0.00	0.9467		
Embryo sex	2, 139	0.33	0.7174	0.51	**0.6034**
MBC*female condition	1, 32.9	1.50	0.2297		
MBC*female fat score	1, 38.3	1.01	0.3203		
MBC* laying order	5,122	0.52	0.7640		
MBC*breeding round	1, 33.3	0.01	0.9341		
MBC*embryo sex	2,139	3.93	**0.0218**	4.49	**0.0127**

General linear mixed model analysis of DHT concentration within egg yolk to investigate the influence of male colour band treatment. Female identity was entered as a random effect, female condition and fat score were entered into two separate models and significant variables are shown in bold.

Appendix 4: Female condition and yolk carotenoids (chapter 2)



The concentration of total carotenoids (µg/g) plotted against the condition of the laying female (calculated from the residuals of mass: tarsus length).

Appendix 5: Total yolk carotenoids (chapter 2)

Variable	df	Full model		Minimal model	
		F	p	F	p
Male band colour (MBC)	1, 49.5	0.15	0.7013		
Female condition	1, 42.2	11.94	**0.0013**	10.92	**0.0018**
Female fat score	1, 35.2	2.39	0.1310		
Laying order	5, 118	11.44	**<0.0001**	10.82	**<0.0001**
Breeding round	1, 42.4	0.18	0.6730		
Embryo sex	2, 134	2.04	0.1343		
MBC*female condition	1, 42.2	0.80	0.3748		
MBC*female fat score	1, 38.3	1.01	0.3203		
MBC* laying order	5, 118	0.91	0.4795		
MBC*breeding round	1, 42.4	0.11	0.7365		
MBC*embryo sex	2, 134	2.36	0.0985		

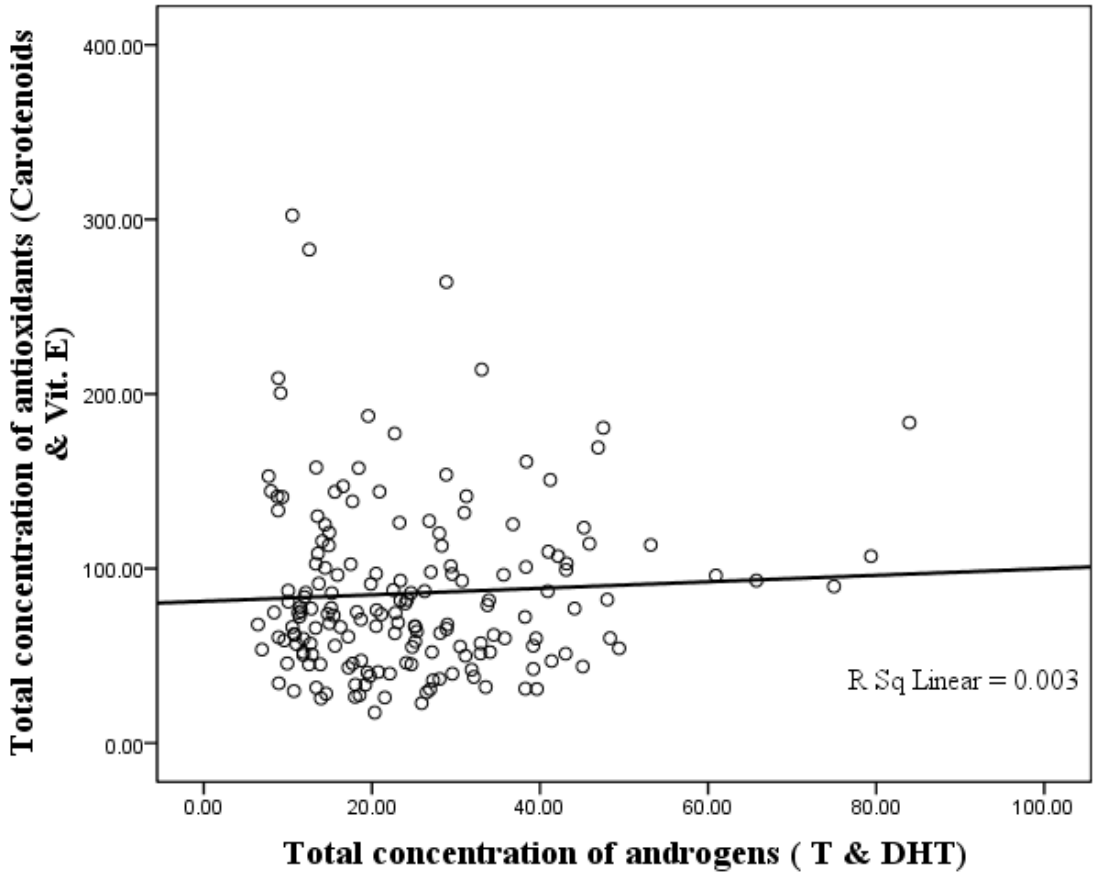
General linear mixed model analysis of total carotenoid concentration within egg yolks, to investigate the influence of male colour band treatment. Female identity was entered as a random effect, female condition and fat score were entered into two separate models and significant variables are shown in bold.

Appendix 6: Vitamin E (chapter 2)

Variable	df	Full model		Minimal model	
		F	p	F	p
Male band colour (MBC)	1, 37.7	0.13	0.7198		
Female condition	1, 38.2	0.27	0.6087		
Female fat score	1, 34.6	0.31	0.5834		
Laying order	5, 93.8	3.79	**0.0036**	3.51	**0.0058**
Breeding round	1, 36.9	1.26	0.2692		
Embryo sex	2, 131	1.44	0.2406		
MBC*female condition	1, 38.2	1.98	0.1679		
MBC*female fat score	1, 34.6	0.38	0.5413		
MBC* laying order	5, 93.8	0.95	0.4529		
MBC*breeding round	1, 36.9	3.47	0.0704		
MBC*embryo sex	2, 131	2.41	0.0935		

General linear mixed model analysis of vitamin E concentration within egg yolks, to investigate the influence of male colour band treatment. Female identity was entered as a random effect, female condition and fat score were entered into models separately and significant variables are shown in bold.

Appendix 7: Relationship between total androgens & total antioxidants (chapter 2)



The total concentration of antioxidants (μg/g) plotted against the total concentration of androgens (pg/mg) within each egg yolk analysed (n = 173).

Appendix 8: Ratio antioxidants: androgens (chapter 2)

Variable	df	Full model		Minimal model	
		F	p	F	p
Male band colour (MBC)	1, 35	0.17	0.6830	0.44	0.5101
Female condition	1, 27.7	8.18	**0.0080**	8.49	**0.0067**
Female fat score	1, 36.8	9.12	0.0046	9.81	**0.0035**
Laying order	5, 86.4	2.17	0.0644		
Breeding round	1, 29.9	0.35	0.5573		
Embryo sex	1, 93.6	0.55	0.4613	0.69	0.4072
MBC*female condition	1, 27.7	0.15	0.6981		
MBC*female fat score	1, 36.8	0.07	0.7966		
MBC* laying order	5, 86.4	1.96	0.0925		
MBC*breeding round	1, 29.9	0.13	0.7177		
MBC*embryo sex	1, 93.6	5.25	**0.0242**	6.42	**0.0127**

General linear mixed model analysis of the ratio of antioxidants: androgens within egg yolks, to investigate the influence of male colour band treatment. Female identity was entered as a random effect, female condition and fat score were entered into models separately and significant variables are shown in bold.

Appendix 9: Sex-ratio (chapter 2)

Variable	df	Full model		Minimal model	
		Wald	p	Wald	p
Male band colour (MBC)	1, 98	0.00	0.9762	1.19	0.2772
Female condition	1, 98	4.82	**0.0305**	3.78	0.0545
Female fat score	1, 98	1.15	0.2859		
Laying order	5, 98	0.79	0.5589		
Breeding round	1, 98	1.45	0.2308		
MBC*female condition	1, 98	9.59	**0.0026**	7.73	**0.0064**
MBC*female fat score	1, 98	0.01	0.9269		
MBC* laying order	5, 98	0.63	0.6791		
MBC*breeding round	1, 98	0.45	0.2308		

Generalized linear mixed model (GLIMMIX) analysis of the primary sex-ratio of clutches to investigate the influence of male colour band treatment. Female identity was entered as a random effect, female condition and fat score were entered into models separately and significant variables are shown in bold. A binomial error distribution was fitted with a logit link function.

Appendix 10: Male song rate in experiment 1 (chapter 3)

Variable	df	Full model		Minimal model	
		F	p	F	p
Male band colour in trial	3, 123	1.59	0.2078		
Male band colour in aviary	2, 123	15.38	**0.0001**	7.27	**0.0010**
Male condition	1, 123	0.03	0.8572		
Female condition	1, 123	0.02	0.8935		
Female response	1, 123	10.26	**0.0017**	5.31	**0.0228**
Trial number	3, 123	1.89	0.1353		

General linear mixed model (GLMM) analysis of male song rate for the no-choice trials conducted in experiment 1. Female identity was entered as a random effect and male identity as a repeated measure.

Appendix 11: Male song rate in experiment 2 (chapter 3)

a)

Variable	df	Full model		Minimal model	
		F	p	F	p
Male band colour in aviary	1, 71	0.01	0.9192		
Male condition	1, 71	7.63	**0.0073**	7.80	**0.0065**
Female condition	1, 71	0.09	0.7699		
Female response	1, 71	35.86	**<0.0001**	40.44	**<0.0001**
Trial number	3, 71	2.56	0.0618		

General linear mixed model (GLMM) analysis of male song rate for the no-choice trials conducted in experiment 2. Female identity was entered as a random effect and male identity as a repeated measure.

b)

Variable	df	Full model		Minimal model	
		F	p	F	p
Male band colour in aviary	1, 72	1.23	0.2713		
Female condition	1, 72	0.34	0.5588		
Female response	1, 72	38.11	**<0.0001**	43.77	**<0.0001**
Trial number	3, 72	2.40	0.0750		

Analysing male song rate (as above), without the addition of male condition to account for any co-linearity between male band colour and male condition.

Appendix 12: Female preferences in experiment 1 (chapter 3)

Variable	df	Full model		Minimal model	
		F	p	F	p
Male band colour in trial	2, 143	2.93	0.0566	2.61	**0.0538**
Male band colour in aviary	1, 143	1.79	0.1826		
Male condition	1, 143	0.12	0.7262		
Female condition	1, 143	0.47	0.4939		
Male song rate	1, 143	16.79	**<0.0001**	14.54	**0.0002**
Trial number	3, 143	7.00	**0.0002**	6.81	**0.0002**

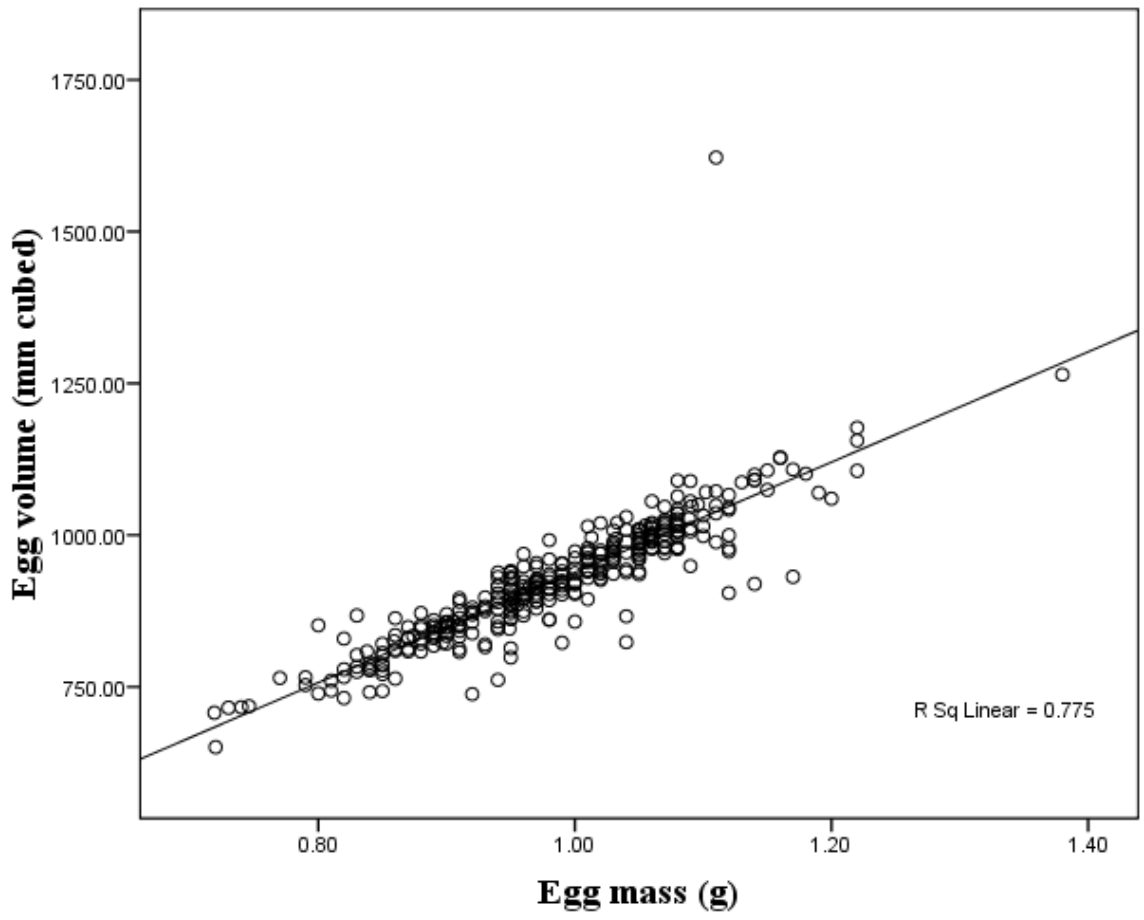
Generalized linear mixed model (GLIMMIX) analysis of female preference for the no-choice trials conducted in experiment 1. Male identity was entered as a random effect and female identity as a repeated measure. The model was fitted to a binomial error distribution with logit-link function.

Appendix 13: Female preferences in experiment 2 (chapter 3)

Variable	df	Full model		Minimal model	
		F	p	F	p
Male band colour in aviary	1, 77	0.17	0.6856		
Male condition	1, 77	0.29	0.5919		
Female condition	1, 77	0.33	0.5658		
Male song rate	1, 77	23.11	**<0.0001**	20.89	**<0.0001**
Trial number	3, 77	1.59	0.1290		

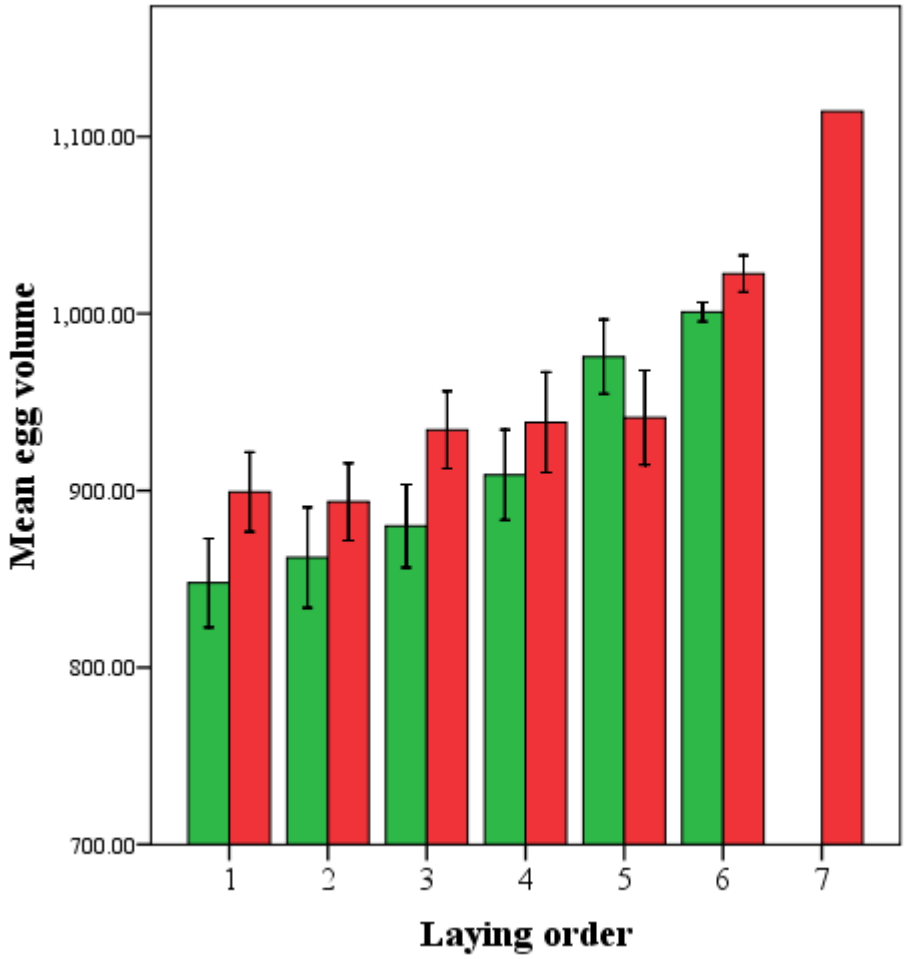
Generalized linear mixed model (GLIMMIX) analysis of female preference for the no-choice trials conducted in experiment 2. Male identity was entered as a random effect and female identity as a repeated measure. The model was fitted to a binomial error distribution with logit-link function.

Appendix 14: Egg mass and volume (chapter 4)



Egg volume (mm^3) plotted against egg mass (grams) for eggs in which both measurements could be made.

Appendix 15: Egg volume and laying order for manipulated male attractiveness experiment (chapter 4)



Mean egg volume in mm^3 (±SE) across the laying order for clutches laid by females that were paired to males that have been give either red of green coloured leg bands.

Appendix 16: Egg volume model for manipulated male attractiveness (chapter 4)

Variable	df	Full model		Minimal model	
		F	p	F	p
Male band colour (MBC)	1, 4.956	0.32	0.5938		
Female condition	1, 4.977	0.80	0.4124		
Female fat score	1, 4.879	3.40	0.1262		
Laying order	6, 62.12	7.11	**<0.0001**	9.96	**<0.0001**
Breeding round	1, 62.06	21.65	**<0.0001**	19.26	**<0.0001**
MBC*female condition	1, 4.977	0.12	0.7477		
MBC*female fat score	1, 4.879	0.13	0.7335		
MBC* laying order	5, 62.16	0.16	0.9747		
MBC*breeding round	1, 62.06	0.03	0.8720		

General linear mixed model analysis of egg volume to investigate the influence of male colour band treatment. Female identity was entered as a random effect, female condition and fat score were entered into two separate models and significant variables are shown in bold.

Appendix 17: Clutch size for manipulated male attractiveness (chapter 4)

Variable	df	Full model		Minimal model	
		Wald	p	Wald	p
Male band colour (MBC)	1, 1	0.19	0.7409		
Female condition	1, 1	0.04	0.8733		
Female fat score	1, 1	0.02	0.9194		
Breeding round	1, 1	0.30	0.6803		
MBC*female condition	1, 1	0.29	0.6867		
MBC*female fat score	1, 1	0.17	0.7521		
MBC* laying order	1, 1	0.30	0.6803		

Generalized linear mixed model (GLIMMIX) analysis of clutch size to investigate the influence of male colour band treatment. Female identity was entered as a random effect, female condition and fat score were entered into models separately and significant variables are shown in bold. A poisson error distribution was fitted with a log link function. All variables were removed from the minimal model.

Appendix 18: Numbers of hatched and fledged chicks (chapter 4)

a) Hatched chicks

Variable	df	Full model		Minimal model	
		F	p	F	p
Male band colour (MBC)	1, 4	0.19	0.6830		
Female condition	1, 4	1.81	0.2499		
Female fat score	1, 4	0.05	0.8418		
MBC*female condition	1, 4	0.00	0.9536		
MBC*female fat	1, 4	1.37	0.3070		

Generalized linear mixed model (GLIMMIX) analysis of numbers of hatched chicks to investigate the influence of male colour band treatment. Female condition and fat score were entered into models separately. A poisson error distribution was fitted with a log link function. All variables were removed from the minimal model.

b) Fledged chicks

Variable	df	Full model		Minimal model	
		F	p	F	p
Male band colour (MBC)	1 , 1	0.01	0.9473		
Female condition	1 , 1	0.47	0.6173		
Female fat score	1 , 1	0.70	0.5566		
MBC*female condition	1, 1	0.03	0.8943		
MBC*female fat	1, 1	0.00	0.9937		

Generalized linear mixed model (GLIMMIX) analysis of numbers of fledged chicks to investigate the influence of male colour band treatment. Female condition and fat score were entered into models separately. A poisson error distribution was fitted with a log link function. All variables were removed from the minimal model.

Appendix 19: Correlations between male phenotypic traits (chapter 4)

		Condition	Fat Score	Beak colour	Cheek patch	Chest patch
Condition	Pearson Correlation	1.000	**.548****	**-.210****	-.038	-.096
	Sig. (2-tailed)		**.000**	**.000**	.573	.157
	N	324.000	**324**	**277**	217	217
Fat score	Pearson Correlation	.548**	1.000	**.135***	-.122	-.070
	Sig. (2-tailed)	.000		**.024**	.074	.303
	N	324	324.000	**277**	217	217
Beak colour (PC1)	Pearson Correlation	**-.210****	**.135***	1.000	-.031	**.413****
	Sig. (2-tailed)	**.000**	**.024**		.691	**.000**
	N	**277**	**277**	299.000	170	**170**
Cheek patch size	Pearson Correlation	-.038	-.122	-.031	1.000	**.262****
	Sig. (2-tailed)	.573	.074	.691		**.000**
	N	217	217	170	217.000	**217**
Chest patch size	Pearson Correlation	-.096	-.070	**.413****	**.262****	1.000
	Sig. (2-tailed)	.157	.303	**.000**	**.000**	
	N	217	217	**170**	**217**	217.000

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Pearson's correlations between all male phenotypic traits measured, significant variables are shown in bold.

Appendix 20: Egg volume with male phenotypic traits (chapter 4)

a)

Variable	df	Full model		Minimal model	
		F	p	F	p
Male fat score	1, 12.6	7.70	**0.0161**	7.50	**0.0096**
Chest patch size	1, 11	1.03	0.3323		
Female condition	1, 11.9	0.58	0.4628		
Female fat score	1, 11.7	0.22	0.6464		
Laying order	1 , 141	73.11	**<0.0001**	47.04	**<0.0001**
Breeding attempt	3 , 147	8.02	**<0.0001**	3.32	**0.0202**

General linear mixed model (proc GLMM) analysis of numbers of egg volume to investigate the influence of male fat score and chest patch size. Female identity was entered as a random effect, female condition and fat score were entered into two separate models and significant variables are shown in bold.

b)

Variable	df	Full model		Minimal model	
		F	p	F	p
Male condition	1, 12.2	0.11	0.7478		
Cheek patch size	1, 11.9	0.04	0.8941		
Female condition	1, 12.2	0.34	0.5699		
Female fat score	1, 11.7	0.04	0.8542		
Laying order	1, 140	74.14	**<0.0001**	47.83	**<0.0001**
Breeding attempt	3, 144	7.39	**0.0001**	3.49	**0.0160**

General linear mixed model analysis of egg volume to investigate the influence of male condition and cheek patch size. Female identity was entered as a random effect, female condition and fat score were entered into two separate models and significant variables are shown in bold.

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Appendix 20 continued.

c)

Variable	df	Full model		Minimal model	
		F	p	F	p
Male beak colour (PC1)	1, 15.6	0.07	0.7898		
Female condition	1, 17	0.24	0.6320		
Female fat score	1, 15.9	0.43	0.5207		
Laying order	1, 159	27.79	**<0.0001**	47.83	**<0.0001**
Breeding attempt	3, 166	3.98	**0.0090**	3.49	**0.0160**

General linear mixed model analysis of egg volume to investigate the influence of male beak colour. Female identity was entered as a random effect, female condition and fat score were entered into two separate models and significant variables are shown in bold.

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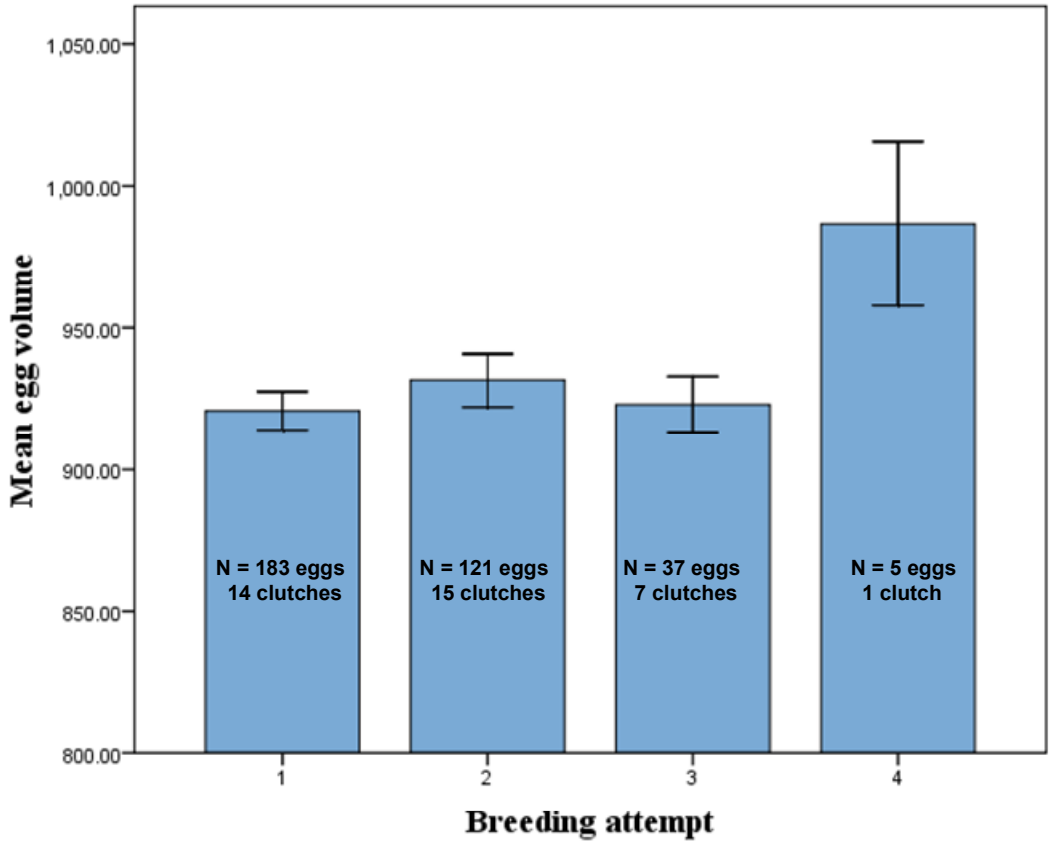
Appendix 21: Egg volume with breeding attempt (chapter 4)



Mean egg volume in mm3 (± SE) for eggs laid within successive breeding attempts by the same female between September – December 2007.

Appendix 22: Yolk T conc. with male phenotypic traits (chapter 4)

a)

Variable	df	Full model		Minimal model	
		F	p	F	p
Male fat score	1, 4.61	0.34	0.5873		
Chest patch size	1, 5.19	6.32	0.0518	5.52	**0.0332**
Female condition	1, 3.99	0.00	0.9816		
Female fat score	1, 4.73	0.60	0.4746		
Laying order	1, 43.3	8.44	**0.0058**	11.25	**0.0013**
Breeding attempt	3, 34.8	0.22	0.8791		

General linear mixed model (proc GLMM) analysis of yolk T concentrations to investigate the influence of male fat score and chest patch size. Female identity was entered as a random effect, female condition and fat score were entered into two separate models and significant variables are shown in bold.

b)

Variable	df	Full model		Minimal model	
		F	p	F	p
Male condition	1, 9.26	0.22	0.6477		
Cheek patch size	1, 9.58	0.40	0.5402		
Female condition	1, 9.89	0.91	0.3616		
Female fat score	1, 8.85	0.01	0.9129		
Laying order	1, 47	8.76	**0.0048**	8.49	**0.0043**
Breeding attempt	3, 51.8	0.22	0.8790		

General linear mixed model analysis yolk testosterone to investigate the influence of male condition and cheek patch size. Female identity was entered as a random effect, female condition and fat score were entered into two separate models and significant variables are shown in bold.

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Appendix 22 continued.

c)

Variable	df	Full model		Minimal model	
		F	p	F	p
Male beak colour (PC1)	1, 69	8.03	**0.0060**	8.95	**0.0034**
Female condition	1, 75	3.02	0.0864		
Female fat score	1, 69	0.69	0.4100		
Laying order	1, 69	6.50	**0.0130**	4.67	**0.0329**
Breeding attempt	3, 69	0.73	0.5377		

General linear mixed model analysis of egg volume to investigate the influence of male beak colour. Female identity was entered as a random effect, female condition and fat score were entered into two separate models and significant variables are shown in bold.

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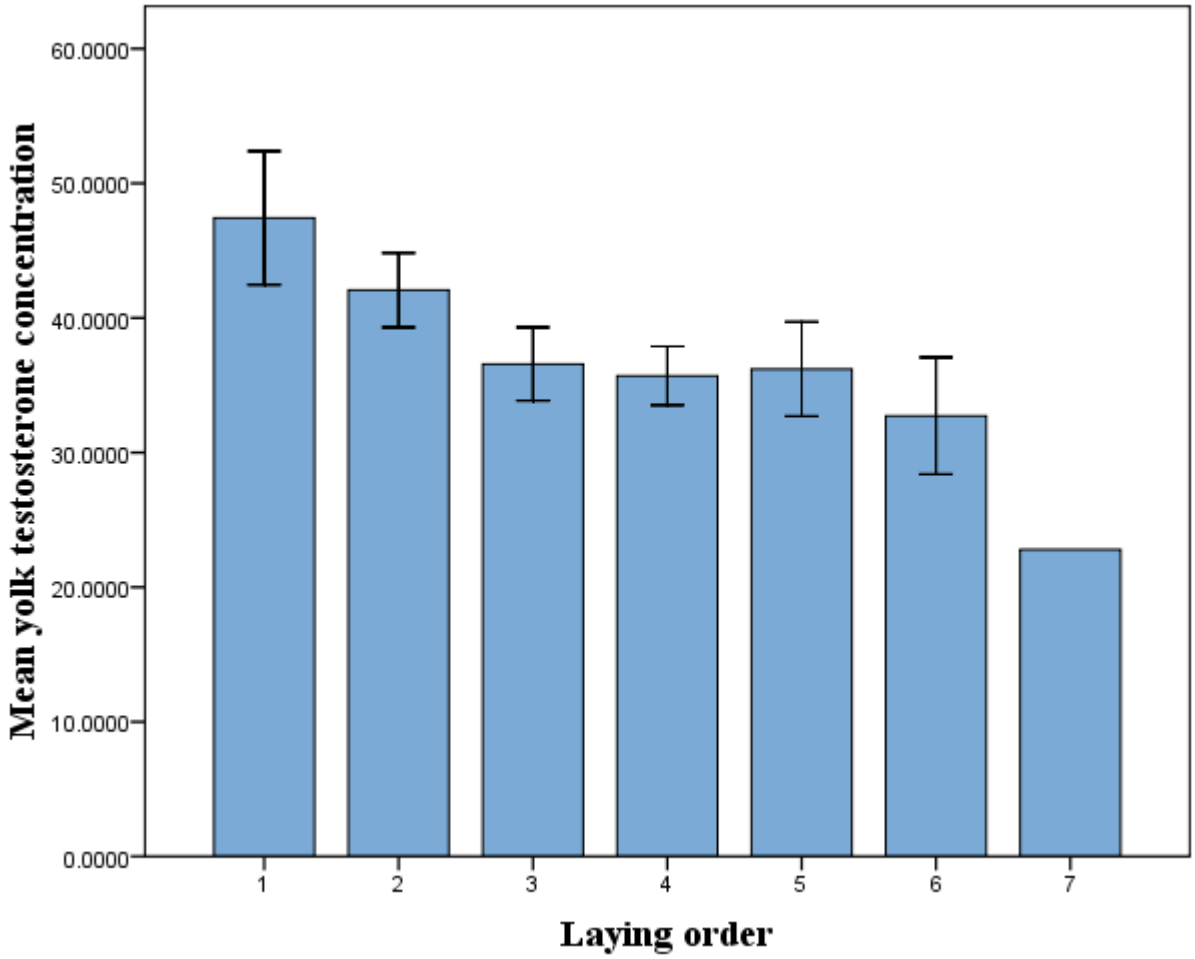
Appendix 23: Yolk T concentration across the laying order (chapter 4)



Mean yolk testosterone concentration in pg/mg (± SE) for eggs across the laying order of clutches laid by wild zebra finches.

Appendix 24: Clutch size with male phenotypic traits (chapter 4)

a)

Variable	df	Full model		Minimal model	
		F	p	F	p
Male fat score	1, 1	0.00	0.9984		
Chest patch size	1, 1	0.00	0.9961		
Female condition	1, 1	0.04	0.8789		
Female fat score	1, 1	0.66	0.5666		

b)

Variable	df	Full model		Minimal model	
		F	p	F	p
Male condition	1, 1	0.38	0.6467		
Cheek patch size	1, 1	0.26	0.7009		
Female condition	1, 1	0.00	0.9645		
Female fat score	1, 1	0.38	0.6490		

c)

Variable	df	Full model		Minimal model	
		F	p	F	p
Male beak colour (PC1)	1, 1	0.00	0.9931		
Female condition	1, 1	0.01	0.9497		
Female fat score	1, 1	1.11	0.4835		

Generalized linear mixed model (proc GLIMMIX) analysis of clutch sizes to investigate the influence of male phenotypic traits. Female identity was entered as a random effect, female condition and fat score were entered into two separate models and a poisson error distribution was fitted.

Appendix 25: Number of hatched chicks with male phenotypic traits (chapter 4)

a)

Variable	df	Full model		Minimal model	
		F	p	F	p
Male fat score	1, 1	3.14	0.3272		
Chest patch size	1, 1	0.10	0.8018		
Female condition	1, 1	2.08	0.3856		
Female fat score	1, 1	2.61	0.3531		

b)

Variable	df	Full model		Minimal model	
		F	p	F	p
Male condition	1 , 1	0.02	0.9089		
Cheek patch size	1 , 1	3.44	0.3147		
Female condition	1, 1	0.00	0.9939		
Female fat score	4, 1	0.88	0.6529		

c)

Variable	df	Full model		Minimal model	
		F	p	F	p
Male beak colour (PC1)	1, 1	2.47	0.3608		
Female condition	1, 1	6.76	0.2337		
Female fat score	-				

Generalized linear mixed model (proc GLIMMIX) analysis of the number of hatched chicks to investigate the influence of male phenotypic traits. Female identity was entered as a random effect, female condition and fat score were entered into two separate models and a poisson error distribution was fitted.

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Appendix 26: Number of fledged chicks with male phenotypic traits (chapter 4)

a)

Variable	df	Full model		Minimal model	
		F	p	F	p
Male fat score	1 , 1	0.01	0.9318		
Chest patch size	1, 1	0.06	0.8446		
Female condition	1, 1	0.07	0.8338		
Female fat score	1, 1	0.28	0.6897		

b)

Variable	df	Full model		Minimal model	
		F	p	F	p
Male condition	1, 1	0.00	0.9924		
Cheek patch size	1, 1	0.44	0.6276		
Female condition	1, 1	0.42	0.6352		
Female fat score	1, 1	0.19	0.7406		

c)

Variable	df	Full model		Minimal model	
		F	p	F	p
Male beak colour (PC1)	1, 1	0.23	0.7140		
Female condition	1, 1	0.14	0.7700		
Female fat score	1, 1	1.36	0.4516		

Generalized linear mixed model (proc GLIMMIX) analysis of the number of fledged chicks to investigate the influence of male phenotypic traits. Female identity was entered as a random effect, female condition and fat score were entered into two separate models and a poisson error distribution was fitted.

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Appendix 27: Hatching mass of chicks with male phenotypic traits (chapter 4)

a)

Variable	df	Full model		Minimal model	
		F	p	F	p
Male fat score	1, 7.152	0.00	0.9862		
Chest patch size	1, 4.469	0.24	0.6482		
Female condition	1, 5.081	0.20	0.6698		
Female fat score	1, 8.001	0.00	0.9893		
Hatching order	4, 8.058	0.86	0.5284		
Breeding attempt	2, 8.528	15.14	**0.0016**	13.97	**<0.0001**

b)

Variable	df	Full model		Minimal model	
		F	p	F	p
Male condition	1, 8.132	5.04	0.0546	3.05	0.0896
Cheek patch size	1, 8	0.04	0.8399		
Female condition	1, 8.064	8.35	0.5685		
Female fat score	1, 1	1.91	0.3988		
Hatching order	4, 8.14	2.62	0.1135		
Breeding attempt	2, 8.104	28.21	0.0002	17.69	**<0.0001**

c)

Variable	df	Full model		Minimal model	
		F	p	F	p
Male beak colour (PC1)	1, 11.71	0.21	0.6548		
Female condition	1, 11.49	5.33	**0.0405**		
Female fat score	1, 11.67	3.40	0.0905		
Hatching order	4, 11.08	3.05	0.0639		
Breeding attempt	3, 11.35	42.25	**<0.0001**	13.97	**<0.0001**

General linear mixed model analysis of chick hatching mass to investigate the influence of male phenotypic traits. Female identity was entered as a random effect, female condition and fat score were entered into two separate models and significant variables are shown in bold.

Appendix 28: Mass of day 10 old chicks with male phenotypic traits (chapter 4)

a)

Variable	df	Full model		Minimal model	
		F	p	F	p
Male fat score	1, 9	0.24	0.6332		
Chest patch size	1, 9	1.73	0.2207		
Female condition	1, 9	3.57	0.0915	7.74	**0.0104**
Female fat score	1, 9	0.32	0.5840		
Hatching order	4, 9	0.04	0.9971		
Breeding attempt	2, 9	0.24	0.7944		

b)

Variable	df	Full model		Minimal model	
		F	p	F	p
Male condition	1, 8.14	0.75	0.4127		
Cheek patch size	1, 8.506	2.72	0.1357		
Female condition	1, 8.556	0.14	0.7156	7.74	**0.0104**
Female fat score	1, 8.312	2.98	0.1212		
Hatching order	4, 8.166	0.02	0.9988		
Breeding attempt	2, 5.887	0.16	0.8528		

c)

Variable	df	Full model		Minimal model	
		F	p	F	p
Male beak colour (PC1)	1, 10.99	5.55	**0.0381**	6.07	**0.0247**
Female condition	1, 10.14	3.44	0.0930	12.48	**0.0027**
Female fat score	1, 8.674	0.23	0.6435		
Hatching order	4, 10.06	0.13	0.9670		
Breeding attempt	2, 10.51	0.59	0.5732		

General linear mixed model analysis of 10 day old chick mass to investigate the influence of male phenotypic traits. Female identity was entered as a random effect, female condition and fat score were entered into two separate models and significant variables are shown in bold.

Appendix 29: Chick immune response with male phenotypic traits (chapter 4)

a)

Variable	df	Full model		Minimal model	
		F	p	F	p
Male fat score	1, 2.017	3.03	0.2229		
Chest patch size	1, 3.083	0.21	0.6783		
Female condition	1, 6.65	7.61	**0.0297**		
Female fat score	1, 4.773	7.91	**0.0394**	15.30	**0.0014**
Hatching order	4, 6.292	8.47	**0.0107**	10.07	**0.0004**
Breeding attempt	2, 5.405	6.10	**0.0412**	5.11	**0.0203**

b)

Variable	df	Full model		Minimal model	
		F	p	F	p
Male condition	1, 7	2.90	0.1324		
Cheek patch size	1, 7	4.17	0.0804		
Female condition	1, 7	1.77	0.2248		
Female fat score	1, 1	0.32	0.6729		
Hatching order	4, 7	9.34	**0.0062**	5.05	**0.0026**
Breeding attempt	2, 7	7.31	**0.0193**		

c)

Variable	df	Full model		Minimal model	
		F	p	F	p
Male beak colour (PC1)	1, 9.586	5.28	**0.0455**	9.23	**0.0103**
Female condition	1, 9.078	4.39	0.0653	6.73	**0.0235**
Female fat score	1, 10	9.72	**0.0109**	15.30	**0.0014**
Hatching order	4, 9.146	10.35	**0.0019**	11.03	**0.0005**
Breeding attempt	2, 9.603	0.69	0.5256		

General linear mixed model analysis of chick immune response (PHA) to investigate the influence of male phenotypic traits. Female identity was entered as a random effect, female condition and fat score were entered into two separate models and significant variables are shown in bold.

Appendix 30: Assortative pairing by size/condition (chapter 4)

Correlations

		Female condition	Male condition	Female fat score	Male fat score	Female tarsus	Male tarsus
Female condition	Pearson Correlation	1.000	.131	.141	-.067	-.015	-.340
	Sig. (2-tailed)		.533	.511	.751	.941	.096
	N	25.000	25	24	25	25	25
Male condition	Pearson Correlation	.131	1.000	.168	.670**	-.125	-.082
	Sig. (2-tailed)	.533		.433	.000	.512	.667
	N	25	30.000	24	30	30	30
Female Fat score	Pearson Correlation	.141	.168	1.000	.159	-.375	-.346
	Sig. (2-tailed)	.511	.433		.457	.071	.098
	N	24	24	24.000	24	24	24
Male fat score	Pearson Correlation	-.067	.670**	.159	1.000	-.289	-.067
	Sig. (2-tailed)	.751	**.000**	.457		.122	.726
	N	25	30	24	30.000	30	30
Female tarsus	Pearson Correlation	-.015	-.125	-.375	-.289	1.000	.340
	Sig. (2-tailed)	.941	.512	.071	.122		.061
	N	25	30	24	30	31.000	31
Male tarsus	Pearson Correlation	-.340	-.082	-.346	-.067	.340	1.000
	Sig. (2-tailed)	.096	.667	.098	.726	.061	
	N	25	30	24	30	31	31.000

**. Correlation is significant at the 0.01 level (2-tailed).

Pearson's bivariate correlations between male and female size and condition measures of wild pairs that bred in the study site and were included in the male phenotypic trait analyses within chapter 4.

Appendix 31: Estimating yolk T concentrations (chapter 5)

Estimates were obtained by fitting a line to the T concentration plotted against laying order for the first whole clutch of eggs that were analysed. The slope of this line was then used, along with the known concentration of the second laid egg, to estimate the T concentration in all other eggs in the second clutch by using the following equations:

$$c_2 = y_2 - m_1 x_2$$

c_2 = the 2nd clutch intercept

y_2 = T concentration of the 2nd egg

m_1= the slope of the line of the 1st clutch

x_2= the egg number 2

Followed by:

$$y_n = m_1 x_n + c_2$$

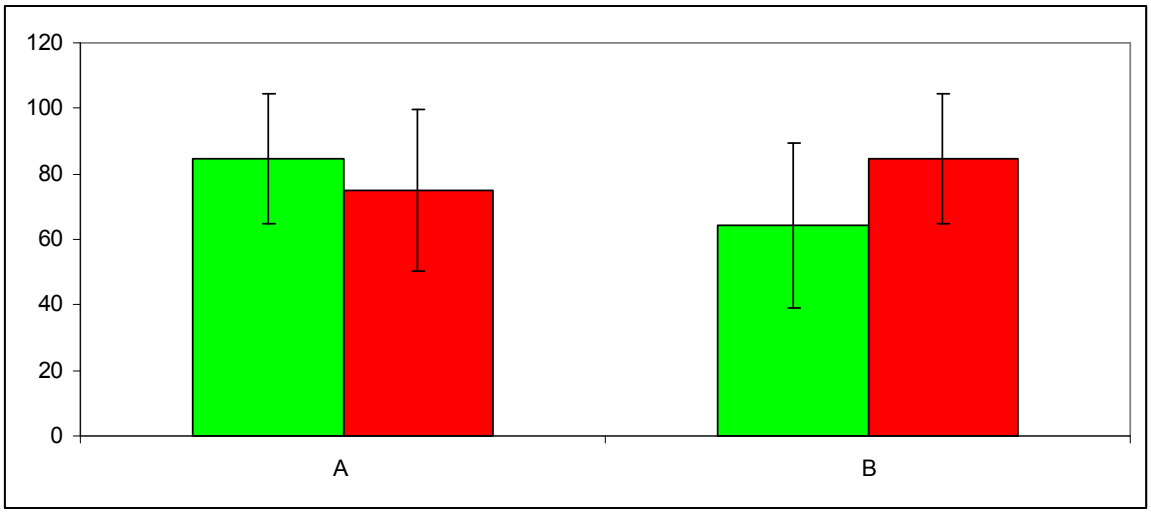
y_n = T concentration of egg number *n*

m_1= the slope of the line of the 1st clutch

x_n= egg number *n*

c_2 = the 2nd clutch intercept

Appendix 32: Percentage of male that paired and reproduced (chapter 5)



Percentage of paired red banded and green banded males in aviary A and B (± 95 % confidence intervals)

Appendix 33: Female traits with male attractiveness (chapter 5)

		df	F	P
Female beak colour	Between Groups	1	.170	.682
	Within Groups	38		
	Total	39		
Female condition	Between Groups	1	.077	.783
	Within Groups	38		
	Total	39		
Female fat score	Between Groups	1	.037	.849
	Within Groups	38		
	Total	39		
Female tarsus length	Between Groups	1	.105	.748
	Within Groups	38		
	Total	39		

One-way ANOVA analysis of female phenotypic traits, comparing between females that initially paired with red banded (attractive) to females that had paired with green banded (unattractive) males.

Appendix 34: Correlations between male and female traits (chapter 5)

		Female condition	Female fat score	Male fat score	Male beak colour	Female beak colour	Male condition
Female Condition	Pearson Correlation	1.000	.385*	.062	-.200	-.311	.215
	Sig. (2-tailed)		**.014**	.705	.216	.051	.183
	N	40.000	40	40	40	40	40
Female fat score	Pearson Correlation	.385*	1.000	.051	.037	-.439**	.036
	Sig. (2-tailed)	**.014**		.754	.823	**.005**	.824
	N	40	40.000	40	40	40	40
Male fat score	Pearson Correlation	.062	.051	1.000	-.082	.107	.444**
	Sig. (2-tailed)	.705	.754		.616	.509	**.004**
	N	40	40	40.000	40	40	40
Male beak colour	Pearson Correlation	-.200	.037	-.082	1.000	.210	-.009
	Sig. (2-tailed)	.216	.823	.616		.193	.954
	N	40	40	40	40.000	40	40
Female beak colour	Pearson Correlation	-.311	-.439**	.107	.210	1.000	-.024
	Sig. (2-tailed)	**.051**	**.005**	.509	.193		.882
	N	40	40	40	40	40.000	40
Male condition	Pearson Correlation	.215	.036	.444**	-.009	-.024	1.000
	Sig. (2-tailed)	.183	.824	**.004**	.954	.882	
	N	40	40	40	40	40	40.000

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Bivariate Pearson's correlations between male and female phenotypic traits of birds that paired and reproduced. Significant variables are shown in bold.

Appendix 35: Egg mass (chapter 5)

Variable	df	Full model		Minimal model	
		F	P	F	P
Male band colour (MBC)	1, 183.2	5.93	**0.0159**	4.91	**0.0279**
Female condition	1, 26.48	0.00	0.9469		
Female fat score	1, 26.34	0.01	0.9435		
Laying order	1, 184.3	1.03	0.3110		
Breeding round	1, 185.4	2.22	0.1377		
Aviary	1, 26.73	0.25	0.6191		
MBC*female condition	1, 184.8	0.25	0.6178		
MBC*female fat score	1, 183.9	0.24	0.6528		
MBC* laying order	1, 183.7	2.49	0.1162		
MBC*breeding round	1, 183.6	2.44	0.1201		
MBC*aviary	1, 184.8	0.34	0.5577		

General linear mixed model analysis of egg weight to investigate the influence of male colour band treatment. Female identity was entered as a random effect, female condition and fat score were entered into models separately and significant variables are shown in bold.

Appendix 36: Yolk T for second laid eggs (chapter 5)

Variable	df	Full model		Minimal model	
		F	P	F	P
Male band colour (MBC)	1, 20.48	0.04	0.8356		
Female condition	1, 23.28	0.45	0.5107		
Female fat score	1, 19.13	0.86	0.3660		
Breeding round	1, 19.7	3.41	0.0798	3.63	0.0687
Aviary	1, 20.08	4.56	**0.0452**	3.84	0.0616
MBC*female condition	1, 22.48	0.39	0.5406		
MBC*female fat score	1 ,18.37	0.29	0.5975		
MBC* breeding round	1, 20.41	0.23	0.6401		
MBC*aviary	1, 19.47	2.46	0.1329		

General linear mixed model analysis of yolk testosterone concentrations in the second laid eggs of two successive clutches, to investigate the influence of male colour band treatment. Female identity was entered as a random effect, female condition and fat score were entered into models separately and significant variables are shown in bold.

Appendix 37: Yolk T for whole clutches (chapter 5)

Variable	df	Full model		Minimal model	
		F	P	F	P
Male band colour (MBC)	1, 45.5	0.00	0.9744		
Female condition	1, 17.74	0.13	0.7242		
Female fat score	1, 18.14	0.20	0.6625		
Laying order	1, 63.49	13.35	**0.0005**	13.66	**0.0004**
Aviary	1, 17.05	6.95	**0.0173**	9.23	**0.0061**
MBC*female condition	1, 17.74	0.41	0.5318		
MBC*female fat score	1, 18.14	0.44	0.5140		
MBC*laying order	1, 63.49	0.01	0.9387		
MBC*aviary	1, 17.05	0.35	0.5642		

General linear mixed model analysis of yolk testosterone concentrations in whole clutches (first laid clutch), to investigate the influence of male colour band treatment. Female identity was entered as a random effect, female condition and fat score were entered into models separately and significant variables are shown in bold.

Appendix 38: Sex ratio (chapter 5)

Variable	df	Full model		Minimal model	
		F	P	F	P
Male band colour (MBC)	1, 1	0.21	0.7246		
Female condition	1, 1	0.84	0.5276		
Female fat score	1, 1	0.31	0.6762		
Laying order	1, 1	0.45	0.6250		
Breeding round	1, 1	0.12	0.7862		
Aviary	1, 1	0.00	0.9879		
MBC*female condition	1, 1	0.50	0.6068		
MBC*female fat score	1, 1	2.34	0.3683		
MBC*laying order	1, 1	0.08	0.8261		
MBC*breeding round	1, 1	0.19	0.7396		
MBC*aviary	1, 1	0.01	0.9331		

Generalized linear mixed model (proc GLIMMIX) analysis of sex ratio to investigate the influence of male colour band treatment. A binomial error distribution with logit link function was fitted. Female identity was entered as a random effect, female condition and fat score were entered into models separately and significant variables are shown in bold.

Appendix 39: Hatching success (chapter 5)

Variable	df	Full model		Minimal model	
		F	P	F	P
Male band colour (MBC)	1, 1	0.14	0.7757		
Female condition	1, 1	0.02	0.9098		
Female fat score	1, 1	1.56	0.4298		
Laying order	1, 1	0.00	0.9610		
Aviary	1, 1	1.52	0.4340		
MBC*female condition	1, 1	0.00	0.9781		
MBC*female fat score	-	-	-		
MBC*laying order	1, 1	1.61	0.4250		
MBC*aviary	1, 1	0.00	0.958		

Generalized linear mixed model (proc GLIMMIX) analysis of hatching success to investigate the influence of male colour band treatment. A binomial error distribution with logit link function was fitted. Female identity was entered as a random effect, female condition and fat score were entered into models separately and significant variables are shown in bold.

Appendix 40: Chick tarsus length at day 11 (chapter 5)

Variable	df	Full model		Minimal model	
		F	P	F	P
Male band colour (MBC)	1, 7.264	6.16	**0.0409**	7.38	**0.0216**
Female condition	1, 6.802	0.94	0.3665		
Female fat score	2, 7.719	1.12	0.3735		
Hatching order	1, 7.517	0.40	0.5451		
Estimated T	1, 12.67	0.82	0.3810		
Aviary	1, 6.827	2.10	0.1920		
Chick sex	1, 8.808	5.20	**0.0491**	5.30	**0.0403**
MBC*chick sex	1, 8.678	8.90	**0.0160**	7.71	**0.0169**

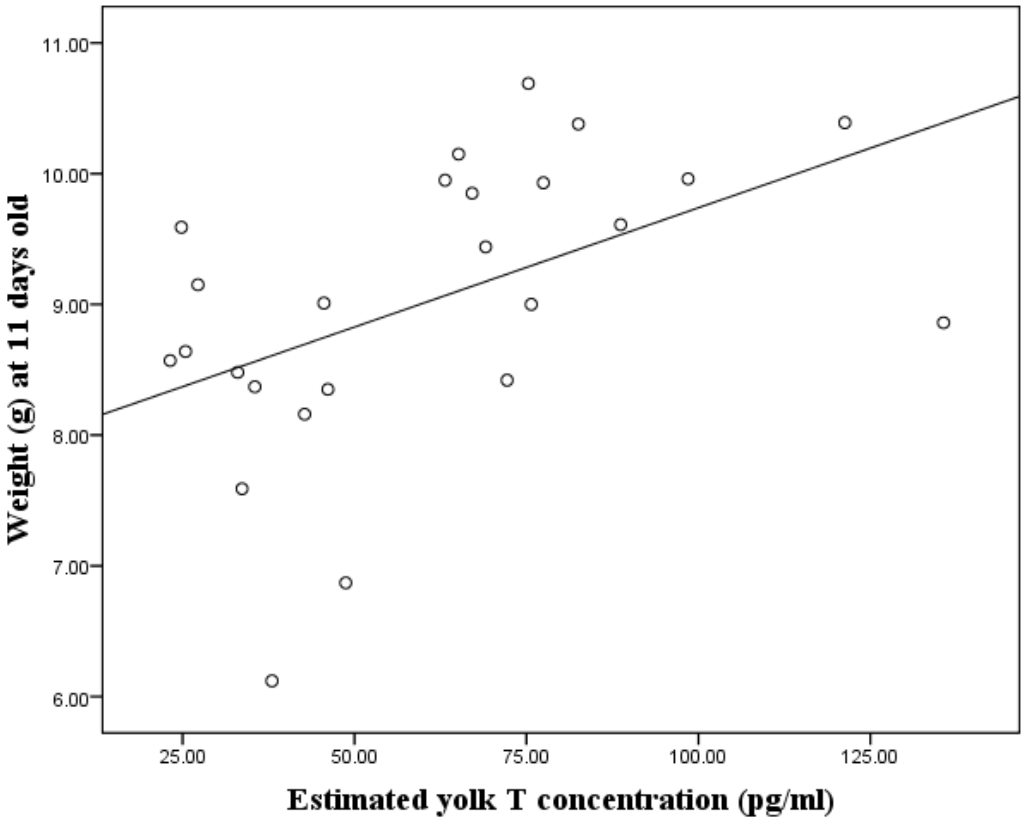
General linear mixed model analysis of chick tarsus length at 10 days old, to investigate the influence of male colour band treatment. Female identity was entered as a random effect, female condition and fat score were entered into models separately and significant variables are shown in bold.

Appendix 41: Chick mass at day 11 (chapter 5)

Variable	df	Full model		Minimal model	
		F	P	F	P
Male band colour (MBC)	1, 9.089	2.58	0.1421		
Female condition	1, 8.749	0.14	0.7208		
Female fat score	1, 8.262	2.72	0.1364		
Hatching order	1, 7.517	17.12	**0.0037**	19.96	**0.0015**
Estimated T	1, 14.93	7.64	**0.0145**	7.58	**0.0127**
Aviary	1, 8.543	2.10	0.1831		
Chick sex	1, 7.823	13.90	**0.0060**	8.27	**0.0187**
MBC*chick sex	1, 7.8	4.20	0.0754		

General linear mixed model analysis of chick mass at 10 days old, to investigate the influence of male colour band treatment. Female identity was entered as a random effect, female condition and fat score were entered into models separately and significant variables are shown in bold.

Appendix 42: Chick mass at day 11 and estimated T conc. (chapter 5)



Weight (in grams) of nestlings at 11 days old plotted against the estimated concentration of T within the yolk, calculated using measured values within the second egg of the clutch and the distribution of allocation of the first laid clutch.

Appendix 43: Chick PHA response (chapter 5)

Variable	df	Full model		Minimal model	
		F	P	F	P
Male band colour (MBC)	1, 6.733	0.03	0.8587		
Female condition	1, 4.009	1.73	0.2581		
Female fat score	1, 5.669	0.07	0.8004		
Hatching order	1, 11.98	0.04	0.8379		
Estimated T	1, 7.369	1.79	0.2202		
Aviary	1, 5.665	1.64	0.2502		
Chick sex	1, 15	0.42	0.5254		
MBC*chick sex	1, 14.88	0.48	0.4979		

General linear mixed model analysis of chick T-cell mediated immune response (PHA injection), to investigate the influence of male colour band treatment. Female identity was entered as a random effect, female condition and fat score were entered into models separately and significant variables are shown in bold.

Appendix 44: Fledging success (chapter 5)

Variable	df	Full model		Minimal model	
		F	P	F	P
Male band colour (MBC)	1, 13.47	0.50	0.4900		
Female condition	1, 12.16	0.11	0.7496		
Female fat score	1, 24	0.90	0.3531		
Hatching order	1, 24	0.86	0.3682		
Aviary	1, 21.57	0.47	0.4998		
Sex of chick	1, 24	0.02	0.8919		
MBC*sex	1, 24	0.82	0.3745		

Generalized linear mixed model (proc GLIMMIX) analysis of fledging success to investigate the influence of male colour band treatment. A binomial error distribution with logit link function was fitted. Female identity was entered as a random effect, female condition and fat score were entered into models separately and significant variables are shown in bold.

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