Maternal Inbreeding Depression in the Zebra Finch, *Taeniopygia guttata*

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Thesis Abstract

The aim of this project was to elucidate the mechanisms behind maternal inbreeding depression, using a model avian species, the zebra finch *Taeniopygia guttata*. Inbreeding can reduce the fitness of inbred animals beyond its negative effects on early survival, through reduced fecundity of inbred animals that survive to reproductive age. In particular, inbreeding has been found to cause a decline in hatching success and early survival of the offspring of inbred mothers. I examined the underlying causes of maternal inbreeding depression by observing the effects of one generation of full-sibling mating on life-history and physiological traits in the zebra finch. The aims of this project were to separate the effects of maternal inbreeding on egg production and subsequent offspring care on the fecundity of inbred females and to examine the possible underlying causes of maternal inbreeding depression.

The study explored the following questions;

- Does maternal inbreeding lead to a reduction in egg production, either in the number, quality or size of eggs produced?

- Do inbred females reduce the level of antimicrobial proteins in their eggs compared to outbred females?

- Does inbreeding lead to a reduction in either incubation attentiveness or incubation temperature in females?

- Does inbreeding in the egg-laying mother lead to a decline in offspring survival or growth?

- Does inbreeding in the foster mother lead to a decline in offspring survival or growth?

- Do inbred birds have higher maintenance costs, i.e. higher resting metabolic rates than outbred females?

After generating inbred and outbred (control) females from full-sibling and non-related pairs respectively, females were paired with unrelated outbred males at the age of around six months old. The first clutch was removed for analysis of egg production (chapter two). The females were immediately allowed to lay replacement clutches, which were cross fostered among nests of inbred and control females. I then compared incubation attentiveness between inbred and control females using this replacement clutch (chapter 3). Through the cross fostering design I was able to separate the effects of inbreeding in the egg laying (chapter 2) and incubating mother (chapter 3) on offspring viability by comparing offspring growth and survival between treatments. When the same group of females were two years old I compared the resting metabolic rate of inbred and control females by measuring oxygen consumption of resting females in an open flow respirometry system (chapter 4).

In chapter two I examined the effects of inbreeding on a key stage of reproductive investment; egg production. I found a reduction in both egg mass and yolk mass in inbred females compared to control females. However, there was little evidence to suggest that the level of antimicrobials deposited to the egg differed with the inbreeding status of the female. Inbreeding in the egg laying mother was found to affect hatchling mass through interactive effects with replicate and clutch size. Inbreeding in the egg egg-laying mother also affected post-hatching survival, although this effect was mediated by hatching order.

In chapter three I investigated the effects of maternal inbreeding on incubation behaviour. Inbred females reduced their incubation attentiveness, but did not reduce average incubation temperature, compared to control females. However, the overall incubation attentiveness experienced by clutches did not differ between treatments due to complete compensation by the partners of inbred females. This is perhaps why there was no significant decline in either hatching success or hatching mass of offspring cross fostered to inbred females.

In chapter four I examined the effects of inbreeding on resting metabolic rate by measuring resting oxygen consumption (VO_2) of inbred females compared to control females. Resting VO_2 (corrected for body size) was higher in inbred compared to control females. Inbred females also showed increased central organ mass (heart plus liver) for their body size compared to control females. Resting VO_2 (corrected for body size) was positively was correlated with central organ mass (corrected for body size) and negatively correlated with

peripheral organ mass (corrected for body size). I also found a positive correlation between resting VO_2 and the ability to evade capture (rank capture order from a flight aviary).

My results suggest that the reduced survival rates of the offspring of inbred females may be caused by reductions in maternal investment, since both egg size/quality and incubation attentiveness have previously been found to positively correlate with offspring viability. The finding that resting VO_2 increased with inbreeding may suggest that inbred females showed reduced maternal investment in egg production and incubation attentiveness due to higher energetic costs of self-maintenance. Resting metabolic rate has been found to be associated with a wide range of life-history traits and so this finding could have important implications for the fitness of inbred animals. These findings are novel and shed light on the previous observations that maternal inbreeding can reduce early and long-term survival of the offspring of inbred individuals in wild populations.

Contents

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Candidate's Declaration

I declare that the work recorded here in this thesis is entirely my own except the recording of resting metabolic rate, which was carried out in collaboration with Shona Smith, as part of her MRes degree at the University of Glasgow and the molecular sexing which was carried out with the help of Kate Griffiths at the University of Glasgow. Experimental design, analysis and interpretation of results were carried out with the help of my supervisors Ruedi Nager and Malcolm Kennedy.

I further declare that that no part of this work has been submitted as part of any other degree.

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1 General Introduction

'It is hardly an exaggeration to say that nature tells us, in the most emphatic manner, that she abhors perpetual self-fertilisation.'

Charles Darwin, 1885

The effects of inbreeding on animal and plant populations have been of central interest to evolutionary biology since Darwin. Recent decades have seen a rapid increase, not only in the understanding of the theoretical underpinnings of inbreeding depression but also in the magnitude of empirical evidence for the consequences of inbreeding, especially in wild populations (Crnokrak & Roff 1999; Hedrick & Kalinowski 2000; Keller & Waller 2002).

Inbreeding can be described in three ways. The first concerns pedigree inbreeding, where the individuals' parents have shared ancestry. The more shared ancestry an individual's parents have, the higher the level of inbreeding. The degree of pedigree inbreeding is calculated by the coefficient of inbreeding *f* (Wright 1922); this is the probability of inheriting two alleles that are identical by decent. The coefficient of inbreeding is half that of the coefficient of relatedness of the parents. The product of full siblings or parent-offspring pairings will therefore be f=0.25, half siblings f=0.125, uncle-niece/aunt-nephew f=0.025 and first cousins f=0.0625. The second measure of inbreeding refers to non-random mating; i.e. if breeding pairs are more closely related than randomly chosen individuals from within the population. This is measured by F_{is} which is the deviation in heterozygosity of an individual from the expected heterozygosity under Hardy-Weinberg equilibrium (reveiwed in Keller & Waller 2002). The third measure concerns inbreeding caused by subdivision between populations. This is calculated from Wright's F_{st} which measures the level of heterozygosity within sub-populations relative to expected heterozygosity under random mating within the entire population (reviewed in Keller & Waller 2002). In general, inbreeding leads to an increase in homozygosity and therefore a decrease in heterozygosity among individuals or populations.

1.1.1 The Prevalence of Inbreeding in Nature

The incidence of inbreeding can vary greatly, often depending on the potential for dispersal, immigration and breeding history. For example, in the Mauritius kestrel *Falco punctatus,* which had previously experienced an extreme population bottleneck, inbreeding was common with around 25% of mating occurring between moderately or closely related individuals, inbreeding levels of $f \geq 0.125$ (Ewing *et al.* 2008). Similarly, in a study of an island population of song sparrows *Melospiza melodia* around 44% of breeding attempts were between known relatives and around 7.6% of all pairings were between close relatives, $f \geq 0.125$ (Keller 1998). A study of two species of island ground finches, *Geospiza scandens* and *Geospiza fortis*, showed moderately high levels of inbreeding. In *G. scandens* 17% of pairings were between known relatives although only 3.3% were between first order relatives, i.e. $f=0.25$. In *G. Fortis* 20% of pairings were between known relatives with only 0.8% between first order relatives (Keller *et al.* 2002). Another study on an island population of great tits *Parus major* estimated moderate levels of inbreeding at around 23% of breeding attempts with around half of these between moderately close relatives $f \geq 0.0625$ (van Noordwijk & Scharloo 1981). By contrast, in a study of a large wild population of the collared flycatcher *Ficedula albicollis*, inbreeding was only detectable in 1.04% of breeding attempts despite an extensive pedigree (Kruuk *et al.* 2002). The frequency of consanguineous mating was also found to be low in a study of great tits, at 1.8%, although over half of these pairings were between full-siblings or mothers and sons. This study probably greatly underestimated inbreeding between more distant relatives as it was based on fairly shallow pedigrees (Bulmer 1973). Greenwood *et al.* (1978) also found very low instances of inbreeding in a great tit population, with 1.5% of all mating occurring between close relatives, $f=0.125$; again, this study could not detect inbreeding between more distant relatives due to shallow pedigrees. A study by Kempenaers *et al.* (1996) on blue tits *Cyanistes caeruleus* showed low levels of inbreeding, with less than 1% of breeding attempts occurring between relatives, although in all of these cases inbreeding was severe, with inbreeding levels of $f=0.25$. As populations become smaller and more isolated, the instances of inbreeding will inevitably increase. Habitat destruction and fragmentation are likely to lead to an increasing frequency of small isolated populations vulnerable to inbreeding (Bawa & Dayanandan 1998).

1.1.2 Inbreeding Avoidance

Inbreeding is often rare in natural populations because many species seem to have evolved mechanisms to avoid mating with close relatives. Inbreeding avoidance may be achieved by recognising and avoiding mates that are closely related or by temporally or spatially avoiding related animals; e.g. sex biased dispersal or delayed maturation (Pusey & Wolf 1996). In mammals, there is evidence that humans and mice *Mus musculus domesticus* avoid mating with close kin by choosing mates with dissimilar MHC genes, apparently based on olfactory cues (reviewed in Penn & Potts 1999). However, in birds the evidence for inbreeding avoidance is mixed (reviewed in Mays *et al.* 2008). A study by Keller & Arcese (1998) on a highly inbred population of song sparrows found little evidence of inbreeding avoidance, with weak evidence for avoidance of parent-offspring pairings but not for other instances of inbreeding. In less isolated populations, one sex may disperse from its natal area to avoid breeding with close kin. In mammals males are the predominantly dispersing sex whereas in birds the female is the predominant disperser (Pusey and Wolfe 1996).

It has been suggested that females may choose a genetically similar male if a related male has a high-quality territory. In these cases, females may seek to increase the genetic diversity of their offspring through extra-pair copulations (reviewed in Mays *et al.* 2008). For example, Freeman-Gallant *et al*. (2006) found that Savannah sparrows *Passerculus sandwichensis* did not appear to choose social mates on the basis of genetic dissimilarity. However, females were more likely to produce extra-pair young if paired with genetically similar males; this generally resulted in extra-pair young that were more heterozygous than within pair young. This could either be the result of females with more closely related mates performing more extra pair copulations or, alternatively, it could be the result of cryptic female choice after copulation. In splendid fairy wrens *Malurus splendens*, a species with exceptionally high levels of extra-pair copulations, levels of extra-pair paternity increased with the amount of genetic similarity between social mates (Tarvin *et al.* 2005). In a study of blue tits Foerster *et al*. (2006) found no evidence for inbreeding avoidance either by social mate choice or extra-pair mate choice; however, fertilization was biased towards the extra-pair male if he was more genetically dissimilar to the female than the social mate. This result suggests that inbreeding avoidance may arise due to cryptic female choice whereby sperm of more distantly related males have a competitive advantage over sperm of more closely related males.

1.2 Inbreeding Depression

1.2.1 The Genetics of Inbreeding Depression

Inbreeding can lead to a reduction in fitness components; this is known as inbreeding depression. Two major mechanisms have been theorised to be responsible for inbreeding depression, the "dominance hypothesis" and the "over-dominance hypothesis" (Charlesworth & Charlesworth 1999). The dominance hypothesis proposes that inbreeding depression is caused by increased expression of the genetic load through unmasking of rare deleterious recessive alleles. The genetic load is the reduction in mean fitness of a population compared to the fitness obtained in a population where all individuals have optimal genomes. This occurs because the chances of inheriting two homologous genes that are identical by decent is increased when related individuals procreate. Therefore, homozygosity for rare deleterious recessive alleles increases, thereby "unmasking" the effects of such genes and leading to the expression of their detrimental effects on the individual's phenotype. Conversely, the overdominance hypothesis posits that inbreeding depression is the result of a reduction in heterozygosity on loci showing heterozygous advantage. There is evidence that both dominance and overdominance effects play a role in inbreeding depression. However, empirical evidence from plant and *Drosophila* research suggests that inbreeding depression is most commonly caused by dominance effects, i.e. expression of recessive or partially recessive deleterious alleles (Charlesworth & Charlesworth 1999; Charlesworth & Willis 2009).

1.2.2 Evidence for Inbreeding Depression

As it is often difficult to measure inbreeding directly due to small pedigrees, some studies instead use measures of heterozygosity at neutral marker loci as a proxy for inbreeding. Many studies have demonstrated correlations between heterozygosity and fitness (reviewed in Chapman *et al.* 2009), despite this, studies have shown that the correlation between heterozygosity and the inbreeding coefficient, *f*, are often weak (Balloux *et al.* 2004; Pemberton 2004; Taylor *et al.* 2010). Two hypotheses have been suggested to explain the observed correlation between heterozygosity and fitness. The general effects hypothesis suggests that heterozygosity at marker loci reflects genome-wide heterozygosity and therefore increased fitness in heterozygotes is caused by dominance or overdominance effects across the genome. However, the local effects hypothesis suggests that heterozygosity-fitness correlations reflect linkage disequilibrium between the marker loci and fitness related genes showing heterozygous advantage (Pemberton 2004;

Acevedo-Whitehouse *et al.* 2009). Heterozygosity fitness correlations therefore do not necessarily show evidence of inbreeding depression and should be interpreted with caution. However, recent studies by Forstmeier *et al.* (2012) and Hemmings *et al.* (2012) in zebra finches, *Taeniopygia guttata*, suggested that heterozygosity may actually be a better reflection of the proportion of the genome that is identical by decent (IBD) than pedigree inbreeding in some cases, given that chance events during meiosis will lead to high variance in the proportion of IBD genes between individuals with the same coefficient of inbreeding. However, the wider implications of this study are still uncertain.

1.2.3 Inbreeding in Life-History Traits vs. Morphological Traits

Inbreeding depression generally impacts more heavily on sexually-selected and life-history traits and has a lesser effect on other traits such as morphology (Houle *et al.* 1996). This is because fitness traits, such as life-history traits and sexually-selected traits are thought to have a different genetic "architecture" compared to traits not closely associated with fitness, resulting in a heightened sensitivity to inbreeding (Rowe & Houle 1996). Genetic architecture is defined as the number of loci and the nature of interactions between loci influencing the trait (Merilä & Sheldon 1999). Fitness traits are hypothesised to be under strong directional selection; however, the strong directional selection that will be present for fitness traits is predicted to reduce the amount of genetic variation in a population, given that alleles bestowing highest fitness should be rapidly driven to fixation, and so strong selection should eventually erode genetic variation, leaving no variation for selection to act upon (Kirkpatrick & Ryan 1991). This could be resolved if we assume that high levels of genetic variance are maintained in fitness traits due to the condition-dependence of such traits, i.e. only animals in good condition are able to maintain high investment in secondary sexual traits or in reproduction (Rowe & Houle 1996; Merilä & Sheldon, 1999). As such condition dependence will result in an increase in the number of loci influencing fitness traits therefore increasing the levels of genetic variance (Rowe & Houle 1996).

A review by Houle *et al.* (1996) on genetic data from a range of animal and plant species showed that, across species, life-history traits had significantly higher mutation rates than morphological traits. This high mutation rate is probably due to the fact that life-history and sexually-selected traits show higher phenotypic variance due to condition dependence. This means that the mutational target for life-history and sexually-selected traits is greater than for other classes of traits due to the large number of loci influencing condition

dependent traits. In accordance with these predictions, a meta-analysis by DeRose & Roff (1999) showed that life-history traits (e.g. survival, fecundity) do in fact show higher inbreeding depression than morphological traits (e.g. body size). Since inbreeding depression is thought to be primarily caused by unmasking of deleterious and partly deleterious mutations (Charlesworth & Charlesworth, 1999; Charlesworth & Willis 2009) this enhanced mutation rate should result in higher levels of inbreeding depression.

1.2.4 Absence of Inbreeding Depression

Despite the detrimental genetic effects of inbreeding, some authors have questioned the importance of inbreeding on the viability of wild populations (e.g. Caro & Laurenson 1994; Caughley 1994). There are many reasons why inbreeding depression may not be detected in natural populations. For example, many studies may lack the statistical power to detect inbreeding depression since levels of inbreeding can often be extremely low (e.g. Bulmer 1973; Greenwood *et al.* 1978; van Noordwijk & Scharloo 1981; Kempenaers *et al.* 1996; Kruuk *et al.* 2002). However, inbreeding depression may not be detected in wild studies for several other reasons, such as historical inbreeding leading to purging of deleterious alleles or environmental dependence of inbreeding depression (Keller & Waller 2002).

1.2.5 Purging of Deleterious Alleles

Previous studies have suggested that inbreeding depression may not be detected because increased expression of deleterious alleles results in "purging" of these alleles from the population (Barrett & Charlesworth 1991). Long-term inbreeding might therefore gradually attenuate inbreeding depression as the population is purged of its genetic load by selection. Fitness may even be predicted to return to (or even exceed) its previous levels after a population bottleneck (Caughley 1994). While viable populations of highly inbred animals have been established (e.g. Chillingham cattle *Bos Taurus*, Visscher *et al.* 2001), inbreeding can lead to extinction of small isolated populations (Saccheri *et al.* 1998). Severe inbreeding may therefore force a population to extinction before it is able to purge its genetic load. Despite the theoretical predictions of genetic purging, inbreeding depression has been observed even within severely bottlenecked populations (Hedrick 1987). A meta-analysis modelled the effects of historical inbreeding on inbreeding depression for neonatal survival in 25 captive mammalian populations (Ballou 1997). This study showed that purging had a consistent, albeit minor, effect on inbreeding depression across populations. A meta-analysis of 99 avian species found no evidence that the genetic

load had been purged from the population as hatching success declined linearly with levels of genetic similarity across species. If purging had occurred the relationship would be predicted to be non-linear since the most highly inbred populations should show relatively high hatching success compared to populations with intermediate levels of inbreeding (Spottiswoode & Møller 2004). Similarly, a study of 22 native New Zealand bird species failed to find any support for purging of deleterious alleles, as hatching success showed a linear decline with increasing severity of population bottleneck. (Briskie & Mackintosh 2004). Furthermore, even highly inbred species such as the naked mole rat *Heterocephalus glaber* appear to have evolved mechanisms for outbreeding (O'Riain *et al.* 1996); this is inconsistent with the idea that populations with high levels of inbreeding can fully alleviate inbreeding depression by purging. It therefore seems likely that purging of the genetic load is unlikely to completely alleviate the effects of inbreeding in nature.

1.2.6 Inbreeding-Environment Interactions

Another reason why inbreeding depression may not be detected is that it can be environmentally dependent. Many studies of birds and mammals have demonstrated that inbreeding fluctuates substantially with environmental conditions; e.g. as food availability, population density, rainfall and parasite load (Coltman *et al.* 1999; Keller *et al.* 2002; Keller & Waller 2002; Brouwer *et al.* 2007). In plants, inbreeding depression of survival in, *Lychnis flos cuculi*, was exacerbated by drought, however there was no effect of drought on inbreeding depression of fecundity (Hausser & Loeschke 1996). In another plant species, *Hydrophyllum appendiculatu,* inbreeding depression was attenuated through maternal effects early in life but increased with age. In the same study, inbreeding depression was found to increase with higher levels of competition (Wolfe 1993). Inbreeding has also been shown to increase in severity between laboratory and natural conditions in white-footed mice *Peromyscus leucopus* (Jiménez *et al.* 1994). However, intriguingly, a study on song sparrows showed that inbreeding depression, manifested as delayed laying date in inbred mothers, was actually lower during cooler years, the same years that the entire population suffered delayed hatching dates (Marr *et al.* 2006). However, the same study also showed that inbred mothers suffered severe hatching failure during wetter years, whereas outbred females were barely affected by rain. This study emphasises the stochastic effects of environment on the severity of inbreeding depression as well as the need for multi-parameter measures in studies of inbreeding depression. A literature review by Armbruster & Reed (2005) attempted to estimate the extent to which inbreeding depression is inflated in stressful environmental conditions. Inbreeding

depression increased under stressful environments in 76% of cases (although this was only significant in 48% of cases). On average, environmental stress increased inbreeding depression by 69% compared to the same intensity of inbreeding in benign environments. Surprisingly, inbreeding depression was actually lower in stressful environments in 24% of cases. This study illustrates that, while there is a tendency for environmental stress to augment inbreeding depression, it is important not to over-generalise this phenomenon.

1.3 Inbreeding and Survival

Inbreeding depression can be manifested across different life-history stages. Firstly inbreeding can affect the zygote of related pairs, resulting in reduced early or long-term survival, or changes in phenotypic traits of the offspring of related individuals, which I will define as inbreeding in the zygote. Secondly, inbreeding can reduce the fecundity of inbred animals by reducing either reproductive output of inbred animals or reduce the survival chances of offspring of inbred animals, which I will define as parental inbreeding.

The most widely observed effect of inbreeding is increased mortality of inbred offspring (Crnokrak & Roff 1999; Keller & Waller 2002). Inbreeding depression is now known to be of particular significance to conservation biology but was not properly studied in endangered populations until the late seventies; a study on small populations of captive ungulates found that juvenile mortality increased in inbred offspring in 15 out of 16 species studied (Ralls *et al.* 1979).

Breeding between close relatives has often been associated with reduced hatching success. An early study of Japanese quail *Coturnix japonica* tested the effects of inbreeding over successive generations in captivity on hatching success of related pairs. For every 10% increment in the inbreeding coefficient, hatching success was reduced by 7% (Sittmann *et al.*1966). There is also a growing body of evidence that mating between close relatives can affect hatching success in wild populations. Breeding between closely related pairs has been found to reduce hatching success in blue tits (Kempenaers *et al.* 1996), collared flycatchers (Kruuk *et al.* 2002), spotless starlings *Sturnus unicolor* (Cordero *et al.* 2004), great reed warblers, *Acrocephalus arundinaceus* (Hansson 2004) and song sparrows (Taylor *et al.* 2010). Reduced hatching success may be a general effect of inbreeding in birds. A meta-analysis by Spottiswoode & Møller (2004) found that species with lower genetic variability (estimated as the band sharing coefficient or percentage of micro-satellite band sharing

among adults) had higher hatching failure. Across the 99 species studied, hatching failure increased from 3.8% to 24% with inbreeding (band sharing ranged between 0.051 and 0.685). Similarly, a study of native New Zealand species found that hatching failure increased with the degree of population bottleneck that the population had experienced (Briskie & Mackintosh 2004). Inbreeding was also found to reduce survival at three early life stages (eyed eggs, alevins and fry) in a farmed population of rainbow trout, *Oncorhynchus mykiss* (Gjerde *et al.* 1983). Hatching success was also found to decline in inbred populations of the butterfly, *Melitaea cinxia* (Saccheri *et al.* 1998). In plants, inbreeding was found to reduce germination rate and survival of *Lychnis flos-cucul* (Hauser & Loeschche 1996). In *Cakile edentula*, selfed plants had lower germination rates and higher rates of seed abortion (Donohue 1998). Inbreeding was found to reduce seed maturation, seed germination and juvenile survival in *Epilobium angustifolium* (Husband & Schemske 1995).

Increased mortality is generally thought to be caused by the heightened expression of lethal mutations under increased homozygosity (Charlesworth & Charlesworth 1999). Some studies have attempted to quantify the effects of inbreeding on survival by estimating the number of "lethal equivalents" that an average individual carries. One lethal equivalent is a combination of selective effects that would have the same impact on the gene pool as one death. A meta-analysis on captive mammalian populations found that the mean number of lethal equivalents was 4.6. This corresponded to a 33% reduction in survival for full-sibling pairings compared to non-sanguineous pairings (Ralls *et al.* 1988). However this study probably underestimates inbreeding depression as it only estimated juvenile survival. A study of a highly inbred population of wild wolves *Canis lupus* found that levels of inbreeding in the pups were highly negatively correlated with winter survival; this corresponded to an estimated 6.04 lethal equivalents (Liberg *et al.* 2005). A study by Bulmer (1973) on a wild population of great tits estimated the number of lethal equivalents based of fledging success as 2.0, although this was based on a very small number of instances of close inbreeding. A study on inbreeding in the New Zealand hihi, *Notiomystis cincta*, found that the average number of lethal equivalents based on fledging success was 6.91 (Brekke *et al.* 2010).

In addition to reducing hatching success and fledging success of inbred offspring, inbreeding can also affect subsequent survival of inbred offspring as demonstrated by the reduced recruitment to the breeding population of inbred song sparrows (Keller 1998), collard flycatchers (Kruuk *et al.* 2002) and Darwin's ground finches *Geospiza fortis and G.*

scandens (Markert *et al.* 2004). Evidence also suggests that inbreeding can reduce lifespan. In the medium ground finch, *Geospiza fortis*, heterozygosity was correlated with increased lifespan (Markert *et al.* 2004) and in the song sparrow inbred individuals had reduced yearly survival compared to more outbred individuals (Keller 1998).

Inbreeding can affect fitness beyond embryonic and juvenile survival as inbred animals often show reduced fecundity, through reduced survival of their own offspring. For example, maternal inbreeding in Holstein cattle was associated with higher rates of late embryonic mortality (Pulkkinen *et al.* 1998). In Botucatu rabbits, inbreeding in the dam reduced the number of offspring born alive (Moura *et al.* 2000). In oilfield mice, *Peromyscus polionotus*, inbred mothers had lower conception rates and lower rates of juvenile survival, compared to control mothers (Margulis & Altmann 1997). A study pigs found that inbreeding in the dam led to lower numbers of piglets born alive (Farkas *et al.* 2007). In Japanese quail, hatching success was reduced by 3% for every 10% increment in inbreeding coefficient of the mother when paired to an unrelated male (Sittmann *et al.* 1966). Maternal inbreeding has been shown to lower hatching success in several wild bird populations. An early study by van Noordwijk & Scharloo (1981) on great tits found that both relatedness between breeding pairs and maternal inbreeding led to a substantial increase in hatching failure. Hatching success was also found to decline with maternal inbreeding in song sparrows (Keller 1998; Marr *et al.* 2006). Egg hatchability was found to follow a quadratic relationship with maternal heterozygosity in spotless starlings. Mothers at intermediate levels of heterozygosity showed the highest hatching success while those with very low or very high levels of heterozygosity showed reduced hatching success (Cordero *et al.* 2004).

Maternal inbreeding has also been associated with reduced fledging success in New Zealand takahe *Porphyrio hochstetteri* (Jamieson *et al.* 2003) and reduced juvenile survival and recruitment during poor seasons in the Seychelles warbler *Acrocephalus sechellensis* (Richardson *et al.* 2004; Brouwer *et al.* 2007). Paternal inbreeding has also been shown to lower offspring success, although this phenomenon has rarely been demonstrated. In a wild population of song sparrows, inbred males fathered fewer nests and also showed lower fledgling survival in their nests (Marr *et al.* 2006). Foerster *et al.* (2003) also found that male heterozygosity was associated with higher fledging success in blue tits.

1.4 Inbreeding and Parental Investment

Some studies have found correlations between parental investment (mainly egg production) and either pedigree inbreeding or heterozygosity, which could help to explain reduce survival of the offspring of inbred parents. Heterozygosity was found to be associated with laying date in great tits (Tomiuk *et al.* 2007). In addition, Marr *et al.* (2006) found an association between pedigree inbreeding and laying date in the song sparrow. Several studies have found heterozygosity to be positively associated with clutch size (Foerster *et al.* 2003; Tomiuk *et al.* 2007; Ortego *et al.* 2007; García-Navas *et al.* 2009; Olano-Marin *et al.* 2011; Wetzel *et al.* 2012). Egg production (number of eggs laid) was found to decline with inbreeding in Japanese quails (Sittmann *et al*. 1966) and white leghorn hens, *Gallus gallus domesticus,* Sewalem *et al.* (1999); these two studies also found a decline in egg volume with inbreeding. Heterozygosity was also associated with egg volume in house sparrows *Passer domesticus* (Wetzel *et al.* 2012) and egg shell spotting in blue tits, which in turn was associated with breeding success (García-Navas *et al.* 2009). Maternal inbreeding was found to influence spawning age and egg number in rainbow trout (Su *et al.* 1996). Ewing (2005) also found reduced provisioning rates in inbred male Mauritius kestrels. However more studies are needed on the effects of inbreeding on parental investment as this may be an important mechanistic cause of maternal inbreeding depression.

1.5 Inbreeding and Immune Function

Evidence linking declines in heterozygosity with reduced immune-competence is beginning to accumulate. Inbred Soay sheep *Ovus aries* showed higher levels of parasitism by gastrointestinal nematodes than their outbred counterparts. This relationship was especially acute in years of high population density. Furthermore, higher parasite burden was associated with increased winter mortality (Coltman *et al.* 1999). In Californian sea lions *Zalophus californianus* inbred individuals were more susceptible to pathogens and showed slower rates of recovery from illness (Acevedo-Whitehouse *et al.* 2006). In a captive population of naked mole rats exposed to a spontaneous outbreak of cornavirus, levels of inbreeding were significantly correlated with mortality rates (Ross-Gillespie *et al.* 2007). However, this relationship is not universal; a study of the rainbow trout found no relationship between genetic diversity and resistance to infection (Overturf *et al.* 2010). In a study of the autumnal moth, *Epirrita autumnata*, inbreeding reduced the immune

response of females but not males (Rantala & Roff 2007). In the Gila topminnow, *Poeciliopsis occidentalis*, mortalities from bacterial infections actually reduced with inbreeding (Giese & Hedrick 2003).

A link between heterozygosity and immunocompetence has also been found in birds. In house finches *Carpodacus mexicanus* individuals with lower heterozygosity experienced higher severity of infection when experimentally exposed to *Mycoplasma gallisepticum* bacteria. Furthermore, heterozygosity showed a significant positive correlation with the response to a phytohaemagglutinin (PHA) challenge injected into the wing web (Hawley *et al.* 2005). PHA is used as a standard measure of cell-mediated immunity (but see Kennedy and Nager 2006 for a review). Genetic diversity across populations of the Galapagos hawk *Buteo galapagoensis* was associated with levels of non-specific natural antibody (NAb), which reflects the innate humeral immune system, and lower parasite abundance (Whiteman *et al.* 2006). A study by Reid *et al.* (2003) found that cell-mediated immunity (PHA swelling) declined in inbred breeding females song sparrows and their chicks. This shows that reduced immune response of inbred females can have important implications for early offspring immunity.

1.6 Inbreeding and Development

Inbreeding between closely related individuals has been seen to inhibit offspring growth in collared flycatchers (Kruuk *et al.* 2002) and Savannah sparrows (Freeman-Gallant 2006). In rainbow trout, adult (but not juvenile) growth was significantly affected by inbreeding (Gjerde *et al.* 1983). In the damselfly *Coenagrion scitulum* inbred males had reduced body size (Carchini *et al*. 2001). Inbreeding depression has also been found to reduce growth in plants (Wolfe 1993; Husband & Schemske 1995; Delph & Lloyd 1996; Sorensen 1999) Inbreeding may also interrupt growth in other ways; inbreeding has been associated with increased fluctuating asymmetry, i.e. deviance from bilateral symmetry due to developmental stress (Clarke *et al.* 1986; Gomendio *et al.* 2000; Bolund *et al.* 2010). There is also evidence that inbreeding may reduce condition; in the European bullhead *Cottus gobio* condition significantly correlated with genetic diversity (which could reflect higher levels of inbreeding) in one of two populations studied (Knaepkens *et al.* 2002). Inbreeding was also found to correlate with poor condition in white-footed mice after reintroduction to the wild (Jiménez *et al.* 1994).

1.7 Inbreeding and Metabolism

Metabolic rate shows additive genetic variance (Rønning *et al*. 2007) and is closely related to fitness as it is associated with both survival (Larivée *et al.* 2010; Artacho & Nespolo 2009; Jackson *et al.* 2001) and reproductive success or expenditure in reproduction (Daan *et al.* 1990; Nilsson & Råberg 2001; Blackmer *et al.* 2005). As such, metabolic traits may be expected to show high levels of inbreeding depression (DeRose & Roff 1999). Some studies have observed a link between heterozygosity and resting metabolic rate (Garton 1984; Rodhouse & Gaffney 1984; Mitton *et al.* 1986; Danzmann *et al.* 1987; Carter *et al.* 1999). Inbreeding has been shown to increase resting metabolic rate in *Gryllodes sigillatus* crickets leading, in turn, to a reduction in endurance due to the higher proportion of energy required for self-maintenance (Ketola & Kotiaho 2009a). Furthermore, sexual signalling was reduced in inbred males in the same population of *G. sigillatus* crickets (Ketola & Kotiaho 2009b). Experimental inbreeding was found to increase the expression of genes involved in metabolism in *Drosophila melanogaster* (Kristensen *et al.* 2005) and so metabolism may be an important underlying cause of inbreeding depression. Furthermore, since resting metabolic rate is associated with a variety of life-history traits, this could have important implications for the reproductive success of inbred females compared to outbred females.

1.8 Life-history of the Zebra Finch, *Taeniopygia guttata*

The zebra finch is a member of the estrildine group of finches, native to Australia and Southeast Asia. The sub-species (and subject of this thesis), *Taeniopygia guttata castanotis*, is native to continental Australia. They are perhaps the most widely studied bird in the laboratory having been exploited in studies on neuroscience, bird song, sexual selection and life-history (Griffith & Buchanan 2010). Recently, the zebra finch genome was sequenced (Stapley *et al.* 2008) making it the first passerine and one of only three bird species to have its genome sequenced (along with the chicken, *Gallus gallus* and the domesticated turkey *Meleagris gallopavo*). Zebra finches can be easily bred in captivity, and reach sexual maturity in captivity at around 3-5 months old. This makes them an excellent species for studying maternal inbreeding in birds.

In the wild, zebra finches feed mostly on grass seeds and therefore their distribution and breeding habits are heavily influenced by precipitation and the abundance of these seeds.

Zebra finches can breed throughout the year, although breeding tends to be concentrated around the spring months when rainfall is highest although reproductive plasticity shows variation between populations (Perfito *et al*. 2007). Zebra finches can therefore be easily encouraged to breed in the laboratory if water and seed is readily available. Zebra finches are socially monogamous with each parent contributing to both incubation, provisioning and brooding of chicks (Burley 1988). At night, however, care is performed exclusively by the female (Zann & Rossetto 1991). Nest building is carried out by both the male and the female; with the male gathering nest material and the female arranging the material within the nest (Zann 1996).

Zebra finches lay around 5 eggs on average (range 2-7). Chicks hatch after around 14 days of incubation, range 11-14 days (Zann 1996). In wild zebra finches, hatching is synchronous; birds begin incubating on the day the last egg is laid in clutches of four eggs or fewer and on the day the fourth egg is laid in clutches of five or more eggs (Zann & Rossetto 1991). However captive birds tend to begin incubation before clutch completion and therefore chicks tend to hatch asynchronously (Rutkowska & Cichon 2005). Chicks fledge at around 18 days-old and reach independence at around 5 weeks of age (Rehling *et al.* 2012). Zebra finches can produce several consecutive broods in a season and so parents will often aggressively encourage the older young to leave the nest in order to start the next brood (Zann 1996).

1.9 Inbreeding in the Zebra Finch

A large number of studies have now been carried on incest avoidance in zebra finches. Slater & Clements (1981) studied the preferences of zebra finches to associate with their own offspring, siblings or non-relatives. They found that mothers and sons associated for significantly more time than unrelated birds. While they found no preferences for fathers and daughters or siblings to associate more than non-kin, overall they found a general preference for the birds to associate with kin over non-kin. A study by Burley *et al.* (1990) found that both male and female zebra finches did not discriminate between siblings and non-kin in mate choice tests. However, females (but not males) showed a preference for first-cousins over non-kin, which may reflect that females are attempting to optimally outbreed. However, these studies did not control for familiarity of kin. A more recent study by Schielzeth *et al.* (2008) used a cross fostering design to ensure that both kin and non-kin were unfamiliar. They found that female zebra finches preferred unrelated males

to brothers in a mate choice trial, however, this result was found in only one of three replicates. These studies are all limited by the fact that they used indirect measures of mate preference and so could simply reflect social preferences, especially given that in most studies females were familiar with their brothers.

Schubert *et al.* (1989) gave female zebra finches a free choice between siblings and non-kin and between siblings and cousins in aviary mate choice tests and found that the number of full-sibling, cousin and non-kin pairs did not differ from the ratios expected from chance. Fetherston & Burley (1990) used a similar design and also found no evidence of inbreeding avoidance again neither of these studies controlled for familiarity among siblings. A study by Arct *et al.* (2010) found that zebra finches were less likely to breed when paired to unfamiliar siblings compared to unfamiliar non-kin. Females also reduced maternal investment when paired to their brothers by laying smaller clutches. The evidence for inbreeding avoidance in zebra finches is inconsistent and suffers from problems with experimental design such as not measuring mate choice directly and not controlling for familiarity with kin. The apparent lack of incest avoidance may reflect low instances of inbreeding in the wild due to, for example, sex biased dispersal (Pusey & Wolf 1996). Although a study by Zann & Runciman (1994) on a wild population of zebra finches found no evidence of sex biased dispersal they did find that colonies were highly fluid, with the majority of birds breeding outside of their natal colony. It is therefore plausible that zebra finches would rarely encounter close kin after sexual maturity in the wild and as such there has been an absence of strong selection for incest avoidance. The lack of incest avoidance in zebra finches will mean that obtaining large number of inbred zebra finches in the laboratory should be a straightforward task.

There have, to date, been relatively few studies of inbreeding depression in the zebra finch. Fetherston & Burley (1990) showed that fledging success was reduced in offspring of full siblings compared to chicks of unrelated birds. Inbred chicks also appeared to have slower development than control chicks as indicated by increased fledging age. Conversely, a more recent study by Bolund *et al.* (2010) found no significant effect of inbreeding on chick survival. However, this study did find significant effects of inbreeding on skeletal size and body mass in both sexes, which persisted into adulthood in females. Inbred females (but not males) showed reduced fat scores (a standard measure of calvicular and abdominal fat) and higher fluctuating asymmetry in the same study and inbred males produced less directed song and had slower syllable rates. The study by Bolund *et al.* also found inbreeding depression for a variety of secondary sexual characteristics; inbreeding

negatively affected bill colour in both males and females (control birds had redder bills than inbred birds). In addition, inbred males showed reduced flank patch size compared to outbred males and were less attractive to females than outbred males in mate choice tests. In the same set of tests inbred females were found to be less active whilst choosing between mates than outbred females. A recent study on zebra finches found significant inbreeding depressions (measured through pedigrees and multi-locus heterozygosity) for 11 phenotypic traits; tarsus length, 8 day old mass, adult mass, bill colour, female choice activity, egg size, female fecundity (number of eggs produced in cages and aviary), male courtship rate, male attractiveness and male siring rate (Forstmeier *et al.* 2012). In addition, a recent study by Hemmings *et al.* (2012) found that inbreeding led to higher rates of embryonic mortality.

Due to the zebra finch's history in captivity, laboratory zebra finches are often assumed to have high levels of ancestral inbreeding. However, a study of 18 captive and two wild populations showed that, while zebra finches have shown some loss of genetic diversity following captivity, they do not appear to have suffered a severe bottleneck. Furthermore, the amount of genetic variation shown in zebra finches was within the range of that found in other studies of wild birds (Forstmeier *et al.* 2007). This suggests that non-inbred zebra finches should present a good baseline with which to study inbreeding as control birds are predicted to have reasonable levels of genetic diversity.

1.10 Aims of Study

As I highlighted in section 1.3, fecundity of inbred individuals and survival of their offspring is often reduced. However, little is known about the underlying mechanisms of maternal inbreeding depression. More studies are needed to investigate the causes of reduced fecundity in inbred females, particularity with respect to the higher rates of mortality that have been found in the offspring of inbred females. One underlying cause of increased mortality of offspring could be reduced maternal investment in inbred females compared to control females. This thesis will investigate maternal inbreeding in zebra finches *Taeniopygia guttata*. By investigating aspects of maternal investment and use of cross fostering studies I will elucidate the causes of maternal inbreeding depression. Furthermore, as resting metabolic rate can have important implications for reproductive success, the thesis will also explore the effect of maternal inbreeding in the zebra finch of

resting VO_2 levels of females. An overview of the experimental design can be found in fig. 1.1, section 1.11.

1.11 Thesis Overview

This thesis will focus on the effects of maternal inbreeding on traits associated with fecundity. While the effect of maternal inbreeding on hatching rate and offspring survival are now well established through laboratory and field studies (particularly in birds) little is known of the underlying causes of this reduction in offspring viability. Experimental laboratory studies provide a powerful method with which to study the underlying causes of maternal inbreeding depression. Laboratory studies allow us to compare breeding success of a large number of highly inbred individuals against control individuals under controlled conditions. In addition, traits closely associated with breeding success such as egg production, incubation effort and provisioning rate can be easily studied in the laboratory. The zebra finch is a useful species for this form of research due to its short generation time and because it is such a well-studied species.

The thesis begins with chapter two which examines the effect of maternal inbreeding on egg production traits. The traits under investigation are; probability to produce a clutch, latency to lay the first clutch, clutch size, egg mass, yolk mass and the concentrations and total amounts of three antimicrobials, lysozyme, ovotransferrin and avidin, deposit to the egg. Through a cross fostering design I also tested whether inbreeding in the egg laying mother affects pre and post-hatching survival and growth in offspring incubated and reared by control females. In chapter three I tested whether inbreeding reduces the incubation attentiveness and incubation temperature and of inbred females compared to control females and if inbreeding in the mother leads to a reduction in the total incubation attentiveness of the pair. In addition, I tested whether offspring from standardised eggs produced by control mothers showed any reduction in embryonic survival and growth when cross fostered and incubated by inbred females compared to offspring raised by control females. In chapter four, I examined the effects of inbreeding on resting metabolic rate of inbred females compared to control females (measured as resting VO_2) to test for a potential increase in maintenance cost of inbred compared to control females. I also compared central and peripheral organ mass of inbred and control females in order to elucidate the underling mechanisms of any such increase in resting VO_2. The same experimental females were used throughout all three experimental chapters, an overview of

the experiments can be seen in fig 1.1 and details of sample sizes and the timing of experiments can be seen in appendix one. In chapter five, I summaries the findings of chapters two, three and four and attempt to set them in the wider context of inbreeding research and suggest the future directions that my findings indicate.

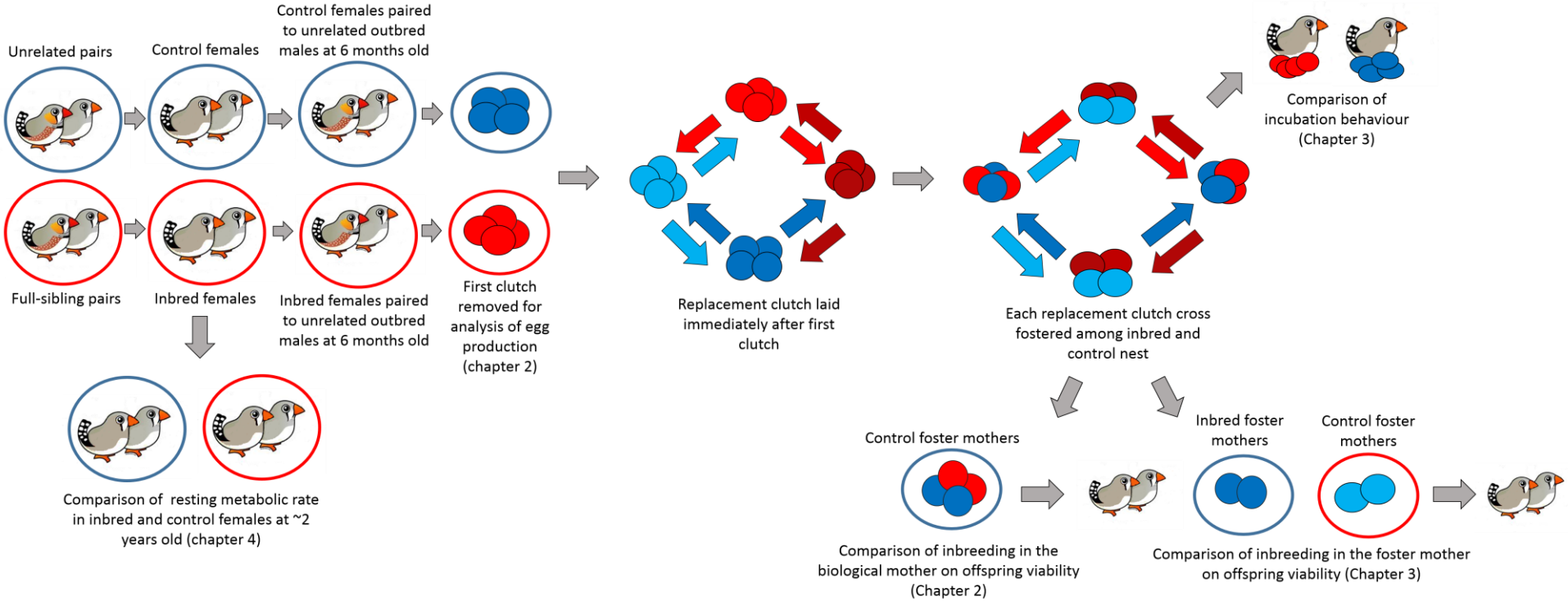


Figure 1.1 Flow diagram of the experimental design. Inbred (red) and outbred (control; blue) females were generated by pairing up full-sibling and non-related pairs, respectively. At the age of around 6 months old, inbred and control females were paired to unrelated outbred males for breeding. The first clutch produced was removed for use in the analysis of egg production (chapter 2). The birds were then immediately allowed to lay a replacement clutch with the same partners; these clutches were cross fostered among nests so that all females incubated a mixture of eggs laid by inbred and control females and no female incubated her own eggs. Incubation behaviour was then compared between inbred and control females on this replacement clutch (chapter 3). The effects of maternal inbreeding on offspring viability was also compared in the replacement clutch of eggs. For the effects of maternal inbreeding on egg production, offspring viability was compared between eggs of inbred and control mothers, but only for eggs incubated by control foster mothers (chapter 2). For the effects of maternal inbreeding in the foster mother, offspring viability was compared between eggs of control mothers only, fostered by either inbred or control mothers. Finally, when the inbred and control females were two years old, their resting metabolic rate was measured and, at the age of around three years, all surviving females were euthanized to obtained measurements of organ size (chapter 4). For details of the sample sizes used and dates of experiments, see appendix one.

2 The Effect of Maternal Inbreeding on Egg Production and Offspring Viability

2.1 Abstract

Inbred female birds often suffer from reduced hatching success and offspring survival; this may be caused by reduced egg quality in inbred individuals. Inbred animals may also show reduced provisioning of egg antimicrobials compared to outbred birds. I investigated whether maternal inbreeding leads to a decline in egg production in terms of clutch size, egg mass and yolk mass. I also tested whether maternal inbreeding leads to a reduction in the level of three antimicrobials transferred to the egg. Finally I used a cross fostering design to determine whether inbreeding in the egg laying mother leads to a decline in offspring viability.

I collected full clutches from 24 inbred (brother x sister) and 26 control nesting captive zebra finch pairs, *Taeniopygia guttata*. I found that egg mass was significantly smaller in inbred females compared to control females; however, this effect was only seen in females that laid below average sized clutches. In addition, yolk mass was also smaller in inbred females although this effect was independent of clutch size. Clutch size was unaffected by inbreeding. There was little evidence that production of antimicrobial proteins was affected by inbreeding. Lysozyme and avidin concentration did not differ between inbred and control females; however, ovotransferrin showed a slight non-significant decline in inbred females. In contrast, total amounts of lysozyme were increased in inbred birds but only in those laying above average clutch sizes. Total amounts of ovotransferrin showed a non-significant decline in inbred females compared to control females while total avidin was not influenced by inbreeding status. In addition to the effects of inbreeding, seasonality also appeared to have a strong effect on egg production, with females breeding in the summer producing larger clutches than females breeding in the autumn.

I then allowed females to lay a replacement clutch in order to study the effects of maternal inbreeding in egg production on offspring viability. Through a cross fostering design I investigated whether inbreeding in the egg laying mother lead to a decrease in offspring viability by comparing hatching success, hatching mass, survival to 35 days and mass at 35 days between offspring produced by inbred and control mothers that were all fostered by

control foster mothers after laying. Mass of hatchlings from eggs laid by inbred and control mothers (fostered control mothers) showed a similar pattern to that of egg mass, although hatchling mass was also affected by replicate. Hatching success was not affected by whether eggs were laid by inbred or control mothers, but post-hatching survival (to day 35) was affected by the interactive effects of inbreeding of the biological mother and brood size.

2.2 Introduction

Inbreeding refers to the phenomenon of breeding between close relatives and leads to an increase in genome-wide homozygosity and changes in mean trait values of offspring compared to offspring of unrelated individuals, known as inbreeding depression (Wright 1977). Inbreeding can result in a reduction in survival and reproductive success (Keller & Waller 2002) and thus is a major ecological and evolutionary factor influencing population dynamics. Although the magnitude of inbreeding depression increases under more stressful environmental conditions (reviewed by Armbruster & Reed 2005), inbreeding depression has been found to be important both under natural conditions and in captive populations (reviewed by Crnokrak & Roff 1999). However, very little is known about the mechanisms by which inbreeding affects fitness.

One fitness component that has been repeatedly reported to be negatively affected by the level of inbreeding of an individual is the survival of their offspring during early development across a broad range of taxa (e.g. Sittmann *et al*. 1966; van Noordwijk & Scharloo 1981; Pulkkinen *et al.* 1998; Su *et al*. 1996; Margulis & Altmann 1997; Keller 1998; Moura *et al.* 2000; Marr *et al.* 2006; Farkas *et al.* 2007). This effect of inbreeding in the parent on survival of their offspring is not well understood, and it had been proposed to be the result of reduced parental expenditure by inbred individuals (Richardson *et al.* 2004). Levels of inbreeding in the parent may adversely influence the amount of care they can provide and thus parental inbreeding can be considered a maternal effect. We have, however, very little empirical evidence of inbreeding depression on parental expenditure that can affect offspring survival. For example, a number of studies in birds have shown increased hatching failure in nests of inbred females independent of offspring inbreeding (e.g. van Noordwijk & Scharloo 1981; Keller 1998; Cordero *et al.* 2004; Ewing 2005; Marr *et al.* 2006). Embryo viability, and thus hatching success, is related to incubation expenditure (e.g. Cook *et al.* 2005; Gorman *et al.* 2005a; Kim & Monaghan 2006; Nord & Nilsson 2011) and the size and quality of eggs (reviewed in Krist 2011). I have shown that inbred females can reduce incubation expenditure (chapter 3.4) however, reduced expenditure on egg production could also be an important underlying cause of parental inbreeding depression in embryo survival.

Inbreeding may be expected to reduce an animal's ability to devote energy to highly demanding activities which may explain the previously observed reductions in egg production in inbred females. The cumulative effect of the expression of deleterious alleles in inbred individuals could reduce their foraging efficiency or efficiency of conversion of energy into fitness-related activities, leading to a reduction in body condition. Indeed, several studies found inbred (or less heterozygous) individuals to be in poorer condition compared to non-inbred (or more homozygous) individuals (Sittmann *et al.* 1966; Danzmann *et al.* 1988; Jiménez *et al.* 1994; Thelen & Allendorf 2001; Knaepkens *et al.* 2002). Individuals with low body condition could have lower resources available for energetically demanding activities such as egg production. Alternatively, inbred individuals may have less energy available to spend on non-self-maintenance activities (Ketola & Kotiaho 2009a). In birds, egg production could be such an energetically and nutritionally demanding activity (Monaghan & Nager 1997; Williams 2005; Nager 2006). If inbred birds have a reduced ability to devote energy to demanding activities, then they may be expected to show reductions in egg production.

Previous studies have suggested that inbreeding may affect egg production. In many studies of inbreeding depression in free-living populations, pedigree information on relatedness among all individuals in a population is not available. Instead, many studies use molecular techniques to estimate genetic heterozygosity of individuals and relate them to measures of fitness, known as heterozygosity-fitness correlations (HFCs), as a proxy for inbreeding depression. Several studies found that birds exhibiting relatively low levels of heterozygosity, that possibly are more inbred, start laying later in the season (Tomiuk *et al.* 2007), lay smaller clutches (Foerster *et al.* 2003; Tomiuk *et al.* 2007; Ortego *et al.* 2007; García-Navas *et al.* 2009; Olano-Marin *et al.* 2011; Wetzel *et al.* 2012) and lay smaller eggs (Wetzel *et al.* 2012). Blue tits, *Cyanistes caeruleus*, exhibiting higher levels of heterozygosity also produced eggs with more even shell spotting which was correlated with higher breeding success and so may reflect egg quality (García-Navas *et al.* 2009). Measures of heterozygosity, however, are often based on a small number of marker loci, and heterozygosity is often only weakly correlated with inbreeding coefficient, *f*, even with a large number of loci, e.g. 100 or more (Balloux *et al.* 2004; Pemberton 2004; Taylor *et al.* 2010). Rather than inbreeding, heterozygosity may instead reflect linkage disequilibrium between the marker loci, and a small number of fitness-related genes showing heterozygous advantage (local effects), rather than genome-wide heterozygosity (general effects) (Pemberton 2004; Acevedo-Whitehouse *et al.* 2006). Studies utilising pedigree inbreeding to investigate inbreeding depression can avoid these issues, although

this is disputed (Forstmeier *et al.* 2012; Hemmings *et al.* 2012). There are, however, only a few studies that have related egg production with inbreeding coefficients derived from pedigrees. Inbred song sparrows, *Melospiza melodia* delayed onset of breeding (Marr *et al.* 2006). Inbred lines of chicken, *Gallus gallus domesticus*, and Japanese quail laid fewer and smaller eggs; however, in these studies it is difficult to disentangle the effects of inbreeding in the dam and inbreeding in the zygote, therefore more research is needed to specifically examine the effects of maternal inbreeding on egg size and egg quality.

Variation in egg production will affect reproductive success. Egg size has been found to correlate with hatchling mass, growth and offspring survival (Krist 2011). Thus, reductions in provisioning to the egg could have important implications for offspring viability. The allocation of resources to the yolk of the egg is important as this contains the main energy store for the developing embryo (Ojanen 1983) as well as important nutrients and hormones (Nager 2006). The effect of maternal inbreeding on yolk size, however, has not previously been investigated. Another important function of some of the egg content is the defence against infections, which can also be resource-demanding (Lochmiller & Deerenberg 2000). An important component of avian offsprings' early innate immunity are antimicrobials deposited by the mother into the albumen of the egg (Tranter & Board 1982), protecting the offspring from microbial infection that can penetrate eggs through the shell (Wellman-Labadie *et al.* 2007) which can cause hatching failure (Cook *et al.* 2005). Therefore, egg antimicrobials are also an important aspect of egg quality. There are three main antimicrobial proteins in the avian egg: lysozyme, ovotransferrin and avidin (Tranter & Board 1982; Shawkey *et al*. 2008). Lysozyme acts as a catalyst for hydrolysis of the cell walls of gram-positive bacteria (Shawkey *et al*. 2008). Ovotransferrin chelates iron, thus reducing the availability of this essential nutrient for bacteria and consequently slowing bacterial growth (Shawkey *et al*. 2008). Avidin binds to biotin, a vitamin essential for bacterial growth, and so also impedes growth in a wide range of bacteria (Wellman-Labadie *et al.* 2007). As the deposition of antimicrobials by the mother to the egg may be limited by its cost and resource availability (Saino *et al.* 2002), and inbred individuals may have a lower resource allocation capacity, I hypothesise lower levels of antimicrobials in eggs of inbred mothers compared to eggs of control females.

One of the difficulties with studying inbreeding depression is that the effects of inbreeding may accumulate too slowly to detect declines in fitness, as close inbreeding tends to be rare and many studies lack high enough statistical power to detect inbreeding depression (Keller & Waller 2002). By studying captive animals, large numbers of inbred animals can

be generated to examine the effects of inbreeding under controlled conditions. Captive zebra finches, *Taeniopygia guttata*, provide a useful system to investigate the effect of maternal inbreeding depression on egg production. Despite their history of captivity, their levels of inbreeding are similar to that of many wild avian populations (Forstmeier *et al.* 2007). Captive zebra finches show similar patterns in their life-history strategies as their free-living counterparts in the wild (Tschirren *et al.* 2009; Griffith *et al.* 2011) and therefore captive zebra finches are reasonably representative of their wild counterparts. I investigated whether inbreeding impairs maternal egg production in the zebra finch. The study explored the following questions;

- Does maternal inbreeding lead to a reduction in egg production, either in the number, mass or quality (i.e. yolk mass) of eggs produced?

- Do inbred females reduce the level of antimicrobial proteins in their eggs compared to outbred females?

- Does inbreeding in the egg-laying mother lead to a decline in offspring survival or growth?

I paired females that resulted from either full-sibling pairings (inbred) or from pairings between unrelated birds (controls), raised at the same time, with unrelated control males. I predicted that if inbred females have a lower resource allocation capacity they would be less likely to produce a clutch, produce smaller clutches, smaller eggs, smaller yolks and/or transfer lower levels of antimicrobial proteins to their eggs. I also compared viability of offspring from eggs produced by inbred and control females by allowing females to lay a replacement clutch that was raised by control foster parents. These results will provide novel insights in which aspects of egg production are affected by inbreeding, and the impact that maternal inbreeding on egg production might have on the viability of offspring from inbred mothers.

2.3 Materials and Methods

2.3.1 Generation of Inbred and Control Lines

The study was carried out in the bird facilities of the University of Glasgow (Law *et al.* 2010). An overview of the experimental design can be seen in figure 1.1, section 1.11 and appendix one. In December 2008 I paired related birds to produce inbred females, and unrelated birds to produce control female zebra finches for our experiment. Breeding pairs were selected from Glasgow University's stock population. The breeding stock at the University of Glasgow consisted of several hundred individuals with known pedigree since 2006; this stock is regularly replenished with birds from other populations (e.g. pet shops) in order to maintain genetic diversity. Prior to the experiment, I supplemented the breeding stock with 10 female zebra finches that were purchased from a local pet shop to minimise potential inbreeding in our stock population; these birds were assumed to be unrelated to any of our stock birds. I set up 19 brother-sister pairs to produce the inbred birds for my experiment. Control birds were bred at the same time and in the same bird room as the inbred birds using 21 pairs between that did not share any parents or grandparents. Pairs were housed in breeding cages (0.4m x 0.6m and 0.4m high) and received the basic breeding diet: *ad libitum* water and mixed dry and soaked seed, and supplemented three times per week with dried hen's egg, vitamins and fresh organic greens (for details see Hill *et al.* 2011). The lighting regime, provided by daylight-spectrum fluorescent tubes (Arcadia Bird Lamp FB36, Redhill, UK), was 12h light: 12h dark. Once paired, birds were provided with nest boxes attached to the side of the cage and coconut fibre for nest building. The likelihood of producing at least one offspring that reached breeding age (5-7 months) did not differ between brother-sister (17 out of 19 pairs) and unrelated pairs (16 out of 21; Fisher's exact test: $p=0.412$). The breeding of experimental birds extended over five months, and several pairs produced several successful broods over that period. At five weeks of age, offspring were separated from their parents and sexes were housed separately in stock cages (0.4m x 1.2m and 0.4 m high) in groups of six birds. Birds were given *ad libitum* access to seed and water and supplemented twice per week with dried hen's egg and greens.

2.3.2 Paring of Inbred and Control Females

From these offspring, I selected the females for my comparison of egg production between inbred and control birds. When the young reached five to seven months of age, I selected females from this group to be paired with unrelated males from the stock population. In the

absence of good quantitative data on inbreeding depression in egg production, I assumed that this would be similar to that of other life-history traits. Using G power (Erdfelder *et al* 1996) I therefore calculated required sample sizes to have an 85% chance of finding a significant difference in egg mass assuming a mean inbreeding depression for life-history traits of 11.8% (DeRose & Roff 1999); this was 28 females per group. Given that I had already decided to pair up 64 females for another part of the study based on a similar power analysis (chapter 3.3.2), I was confident of detecting inbreeding depression in egg production, if present. I selected 32 inbred females (from 17 successful brother-sister pairs) and 32 control females (from 16 successful control pairs that did not share any parents or grandparents). In order to maximise genetic variability among groups I took at least one female from each family; where more than one female from a family was selected, I chose, where possible, females from different broods. Where there was a choice of females I randomly selected a ring number from the spread sheet, using a random number generator. As the pairs that produced the experimental birds bred over an extended period of time, and thus experimental birds varied in age, I divided the experimental females into two replicates so that all females were between five and seven months old when paired for breeding. Replicate one was bred in July 2009 and replicate two in October 2009. The age of females at pairing did not differ between inbred (median=6.3 months, inter quartile range (IQR)=6.2-7.3) and control females (median=6.2, IQR=6.4-6.5; Mann Whitney test, W=1141.5, n=64, p=0.175). The median age of the males that females were paired with did not differ between inbred and control groups (inbred group: median=6.3 months, IQR=6.2-27.6; control group: median=6.3 months IQR=6.3-28.7; Mann Whitney test, W=222, n=64, p=0.113).

The full-sibling pairs and the unrelated pairs produced up to three broods, females in the second replicate were more likely to be from the second or third brood than those in the first replicate (considering only females where incubation data could be collected, the first replicate contained 13 first brood females and 1 second brood female whilst the second replicate contained 5 first brood females, 7 second brood females and 3 third brood females; Fisher's exact test: $p<0.001$). However, the proportion of females from first, second and third broods did not differ between inbred and control group (inbred: 10 first brood females, 3 second brood females and 1 first brood female; control: 8 first brood females 5 second brood females and 2 third brood females; Fisher's exact test: p=0.550).

On the day of pairing all females were weighed to the nearest 0.01g. Pairs were housed in large breeding cages (0.4m x 1.2m and 0.4m high). They received *ad libitum* water during

the experiment, 8 g of soaked mixed seed per day scattered on the floor of the cage and *ad libitum* millet sprays hanging from the ceiling of the cage that could only be reached by flying (Law *et al*. 2010). The larger cage size and scatter feeding of seed in conjunction with the hanging millet spays was intended to increase foraging effort throughout the experiment (G. Law and R.G. Nager unpublished data). Their seed diet was supplemented with dried hen's egg, vitamins and fresh organic greens once a week, and *ad libitum* cuttlefish bone and grit. Each pair was provided with a nest box and a supply of nesting material (coconut fibres). Nest boxes were checked daily for the presence of eggs. Latency to lay was recorded as the number of days between the day that birds were paired for breeding and the day of laying of the first egg of the first clutch. A clutch was defined as two or more eggs laid within a nest and subsequently incubated by the birds. Any clutches that were not incubated were not included in the analysis. Every morning fresh eggs were collected and labelled with a permanent marker to indicate laying female ID and position in the laying sequence, weighed and stored for further measurements (see below "Assessment of Egg Production"). I replaced the collected eggs with a dummy egg made of Fimo clay (Staedtler, Nürnberg, Germany) in order to minimise disturbance of the birds' normal laying behaviour (Haywood 1993).

2.3.3 Offspring Viability

In order to compare offspring viability between offspring from eggs produced by inbred and control mothers, I allowed all experimental females to lay a second clutch and these eggs were cross fostered to be reared before the onset of incubation by a standardised group of control parents (Fig 1.1, section 1.11, Fig. 2.1). Each egg was marked with the nest of origin and laying position. Half of the eggs in each nest were exchanged with half of the eggs from a nest of the same treatment group, and half the eggs were exchanged with half the eggs from a nest of the opposite treatment group. The cross-fostering design ensured that eggs from each treatment were evenly distributed between inbred and control foster parents, and that no egg was raised by its biological parent. I also ensured that pairs always incubated the same number of eggs as they had laid. Nests were checked several times daily for presence of hatchlings and, upon hatching, chicks were weighed to the nearest 0.001g and the downy feathers and legs were marked with permanent marker. The egg of origin was identified by process of elimination as eggs hatched over asynchronously. Chicks were re-coloured and weighed every two days to the nearest 0.001g until 16 days old and then weighed again at 35 days old (independence). Any

unhatched eggs or dead chicks were recorded, and a small tissue sample removed and stored in 100% ethanol at -80^{o}C for molecular sex determination.

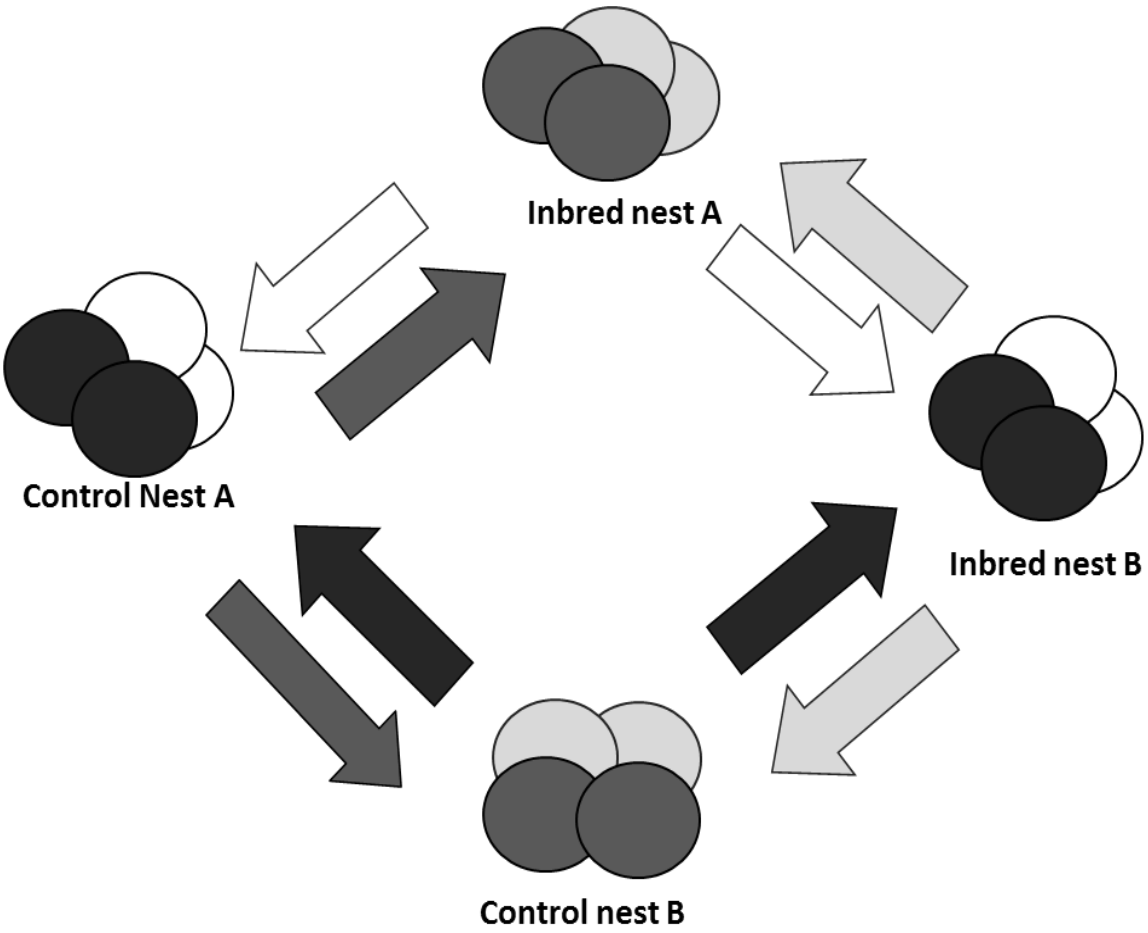


Figure 2.1: Schematic diagram of the cross fostering design. Eggs of inbred mothers are indicated in □ and ■, eggs of control mothers are indicated in ■ and ■. Eggs of each nest were equally divided among two nests of similar laying dates (within 2 days), with one nest belonging to the same treatment group and one nest from the opposite treatment group.

2.3.4 Assessment of Egg Quality

To measure egg composition, I separated the egg into shell, yolk and albumen and weighed each component. Eggs were opened and their contents poured into an aluminium bowl and excess albumen removed from the yolk by running it round the side of the bowl. Wet mass of the isolated yolk was measured to the nearest 0.001 g. The egg shell was air dried to constant weight for 24 hours at room temperature and then weighed to the nearest 0.001 g. Wet albumen mass was estimated by subtracting wet yolk mass and dry shell mass from the fresh egg mass. From each egg, as much albumen as possible was stored at -80^{o}C for antimicrobial protein assays.

To determine if the level of antimicrobial protection transferred from mother to offspring differed between inbred and control females, I measured concentrations of the three most important antimicrobial proteins; lysozyme, ovotransferrin and avidin in the albumen. The concentration of lysozyme in egg albumen was measured following the protocol of Shawkey *et al*. (2008) which was based on earlier work by Osserman & Lawlor (1966). Serial dilutions of standard lysozyme (Fluka, cat no. 62970) were made using phosphate buffer saline (concentration range: 1 mg to 0.0156 mg lysozyme per 100 ml buffer) to derive a standard curve. Buffer was made up of one part 9.5 g/l sodium phosphate dibasic (Sigma, S5136) to 2 parts 9.1 g/l monobasic potassium phosphate (Sigma, P9791). The serial dilution (10 µl per well) was added in triplicate to the first three columns of a 96 well plate (Greiner). The remaining wells contained 10 µl of egg albumen samples in triplicate. Dried *Micrococcus lysodeikticus* bacterial cells (50mg) (Sigma, M3770) were added to 100 ml of 1% agarose solution (Sigma, A0169) at 50-60^{o}C and 150 µl of this this agarose/bacteria mix was added to each well, the plate was left to incubate at 20^{o}C for 3 hours and was scanned at 450 nm to measure light scatter by absorbance for each well using a spectrophotometer (Thermo Scientific multiscan spectrum, Asheville, NC, USA).

Concentration of ovotransferrin in egg albumen was measured following the protocol of Shawkey *et al.* (2008) which was based on earlier work by Yamanishi *et al.* (2002). Serial dilutions of standard ovotransferrin (conalbumen, Sigma, C7786) were made using distilled water (concentration range 100 mg/ml to 1.56 mg/ml). The serial dilutions of ovotransferrin (10 µl per well) were added to the first 7 wells of the first column of a 96 well plate; the final well contained 10 µl of water. Each albumen sample (10 µl per well) was added in triplicate to the rest of the wells. To each well was added 125 µl of a reagent that contained 2ml iron standard solution (Fluka, 16596), 36.34 g of 300 mmol/L Tris

(Sigma Aldrich, 25,285-9), 12.6 g of sodium hydrogen bicarbonate (Sigma, S6014) and 4.2 g of 1 Triton X-100 (Sigma, X100) in 1 litre distilled water. The plate was then incubated for 5 minutes at 37^{o}C. Following this, 25 µl of a reagent that contained 4.92 g of ferrozine (Sigma, P5338), 5.74 g of 32.6 mmol/L l- L-ascorbic acid (Sigma Aldrich, 25,556-4) and 6.05g of Tris (Sigma Aldrich, 25,285-9) in 1 litre distilled water was added to each well. The plate was then incubated for a further 5 minutes at 37^{o}C. Finally, 25 µl of a reagent that contained 115.2 g of 600 mmol/l citric acid (Sigma, C0759) and 1.95 g 25.6 mmol/l thiourea (Fluka, 88810) in 1 litre of distilled water was added to each well. Absorbance of each well was measured immediately by scanning with a spectrophotometer at 570 nm and 660 nm every 20 seconds for a period of 6 minutes and 20 seconds. For each sample the absorbance at 570/660 at the start of the reading period was subtracted from the absorbance at 570/660 at the end of the reading period.

Concentrations of avidin in egg albumen was measured using a colorimetric method following a modified version of Shawkey *et al.* (2008) based on an earlier protocol by Gan and Marquardt (1999). There were insufficient quantities of albumen to carry out measurements for individual eggs since this assay required much larger amounts of albumen than the other two assays. I therefore mixed albumen from all eggs of entire clutches from individual females in order to obtain mean values for each female. Where there was enough albumen available, samples were carried out in triplicate. In total, pooled albumen samples of 43 females were tested: 13 in triplicate (8 inbred and 5 control), 11 in duplicate (5 inbred and 6 control) and 19 singly (6 inbred and 13 control. Sample numbers did not differ between inbred and control females (χ^2 test, χ^2_1=2.82, p=0.244). Samples of 100 µl albumen were added to wells of a 96 well plate, except to the final row, which contained a serial dilution of avidin (Sigma, A9275) with concentration ranging from 5µg/ml to 0.02µg/ml in distilled water. Plates were incubated overnight at 4^{o}C and then rinsed 3 times with phosphate buffered-saline (PBS, Sigma P4417)/0.05% Tween-20, Sigma, P1379). Non-specific binding was inhibited by adding Superblock buffer (Thermo Scientific, 37517) and incubating for 30 seconds at room temperature three times. A 1:4000 solution of biotin-horseradish peroxidase conjugate (Calbiochem, 203194) in Superblock buffer/0.05% Tween-20 was added to each well (100 µl per well) and incubated for 25 minutes at room temperature. The plates were then washed 5 times with PBS/0.05% Tween-20. A solution of TMB (3,3',5,5' tetra methlybenzidine) liquid chromogen (Sigma Aldrich, T8665) was then added to each well (100 µl per well) and the plate was incubated for a further 30 minutes at room temperature. The absorbance of wells was immediately read at 450 nm using a spectrophotometer.

Concentrations of the three antimicrobials were calculated for the linear portion of the standard curve of dilutions of standard antimicrobial vs. absorbance. Repeatability was calculated according to the method by Lessells & Boag (1987) using the following equation:

$R=S^2_A/(S^2_+ S^2_A)$

Where S^2_A is the among-groups variance component and S^2 is the within groups component calculated from mean squares among and between groups (MS_A and MS_W) in the analysis of variance where $S^2 = MS_W$ and $S^2_A = (MS_A – MS_W)$ /number of replicates per sample. Inter-plate repeatability was r=0.54 for lysozyme and r=0.69 for ovotransferrin (avidin samples were all carried out on the same plate). To account for differences between plates, I calculated the ratio between the mean concentration of each plate and the mean concentration of all plates. I then multiplied each value by the ratio of its plate to produce standardised values across plates. This was carried out because readings for the standard mix added to each plate were not consistent. Intra-plate repeatability was r=0.61 for lysozyme, r=0.65 for ovotransferrin and r=0.42 for avidin.

As well as the concentration, I also calculated the total amounts of antimicrobial proteins deposited per egg. Albumen mass was converted to albumen volume using a conversion factor of 970ml/mg (convert-to.com/724/chicken-egg-whites-conversion-plus-nutrients-values.html). Total amounts of antimicrobial proteins in each egg were then calculated by multiplying albumen volume by the concentration of the antimicrobial found for the egg. In the case of avidin, as individual egg samples were pooled for each female, mean albumen volume was multiplied by the mean concentration of avidin found for each female.

2.3.5 Sex Determination

All offspring that reached 35 days of age were sexed according to plumage characteristics. Any offspring that died before this stage were sexed using molecular sex determination from tissue samples. DNA was extracted from tissues using DNeasy Blood & Tissue Kit (Quigen) and polymerase chain reaction (PCR) with primers P2 and P17 were used to amplify regions of the highly conserved chromodomain-helicase-DNA-binding protein (CHD-W), W-linked and chromodomain-helicase-DNA-binding protein (CHD-Z), Z-linked, genes (Arnold *et al.* 2003). I used electrophoresis to distinguish males (a single

CHD-Z band) from females (both CHD- Z and CHD-W bands) with reference to control DNA samples of know sexes (see appendix two for more details). Overall, over 98% of chicks were successfully sexed.

2.3.6 Statistical Analysis

I calculated inbreeding depression in two ways. Firstly, I calculated the coefficient of inbreeding (δ), the percentage change in trait value between control and inbred females for a change in inbreeding coefficient of f=0.25. This is conventionally performed in studies of inbreeding (e.g. DeRose & Roff 1999) and so allows easy comparison of inbreeding depression with previous studies. I also calculated the effect size (Cohen's d) as the difference in trait value divided by the pooled standard deviation of the samples; this gives a standardised measure of inbreeding depression that can be more readily compared across different traits.

Experimental females differed in their age and the brood number they originated from. To breed females at a similar age, they were bred in two replicates, however females from the second replicate were more likely to have hatched from the second or third brood than females in the first replicate (Fisher's Exact test: $p<0.001$). The distribution between first, second and third broods, however, did not differ between inbred and control females (Fisher's Exact test: $p=0.372$). Initial univariate analysis showed no effect of brood number on any aspect of egg production, whereas females in the two replicates differed in clutch size (shown section 2.4), and therefore only the effect replicate was used in the statistical analyses.

Body mass of inbred and control female was compared using a general linear mixed model including inbreeding status and replicate as fixed factors and family as a random factor, as some of the females had the same parents. Latency to lay was analysed using a generalised linear model including inbreeding status and replicate as fixed factors with a quasi-Poisson distribution (due to over dispersion in the initial Poisson distribution model), but it was not possible to include random terms for this model as these are not supported when using the quasi-Poisson error distribution. Clutch size, egg and yolk mass were analysed using general linear mixed models including inbreeding status and replicate as fixed factors and latency to lay (log transformed to correct the left-skew in this variable) as a covariate along with family as a random factor. The models for egg and yolk mass also included female ID nested within family as an additional random factor to account for multiple eggs from the

same female. Female body mass (egg mass generally increases with female body mass; Christians 2002), clutch size (egg mass can vary with clutch size; Christians 2002) and relative laying order (egg mass generally increases throughout the laying sequence in zebra finches; Griffith and Buchanan 2010; Griffith *et al.* 2011) were included as addition covariates in the egg mass and yolk mass models. Relative laying order was calculated by dividing the number of the egg in the laying sequence by the clutch size in order to avoid co-linearity between egg number and clutch size as only largest clutches would have the highest absolute egg numbers. Yolk mass was analysed separately as it contains the majority of the energy content of the egg, but does not necessarily scale with egg size (Williams 1994). All analyses were carried out using *lme* function in the *nlme* package of R 2.12.1 (R Core Development Team 2008).

I could not use principle component analysis to reduce the three antimicrobial proteins into principal components as there were no significant correlations among any of the three antimicrobials ($r \leq 0.171$, $p \geq 0.710$) and the variance was equally distributed among the three principle components (pc1=0.363, pc2=0.333, pc3=0.305). Thus, the concentration of the three antimicrobial proteins varied between eggs and clutches independently of each other and I therefore had to analyse each of them separately. Concentrations and absolute amounts of lysozyme and ovotransferrin were analysed using general linear mixed models with female ID as a random effect nested within family. The following explanatory variables were included in the models: inbreeding status, replicate, clutch size, relative position in laying sequence (adding a quadratic term to test for any non-linear effect of laying sequence as found by D'Alba *et al.* (2010)) and latency to lay (log transformed; concentrations of antimicrobial proteins can vary across the season; Bonisoli-Alquati *et al.*, (2010)). For avidin, there was only one measurement per female from pooled egg samples and I therefore used a general linear mixed model containing family as the only random effect, and inbreeding status, replicate, clutch size and latency to lay (log transformed) as explanatory variables.

Hatching success, post-hatching survival (from hatching to 35 days post hatch), hatchling mass and mass at day 35 were analysed only for chicks that were fostered to control mothers in order to compare offspring viability and growth of offspring from inbred and control eggs raised under standardised conditions. Hatching success and post-hatching survival were analysed using a generalised linear mixed model with binomial error distribution (0=unsuccessful, 1=successful). Inbreeding status of the biological mother and replicate were included as fixed factors and clutch size (brood size at hatching in the case

of chick survival) and relative position in laying/hatching sequence as covariates and biological mother's ID and foster mother's ID were included as crossed random factors. In addition, sex was included as a fixed factor in the model of post-hatching survival. Hatchling mass and mass at day 35 were analysed using general mixed linear models including inbreeding status of the biological mother and replicate as fixed factors and clutch size/brood size and relative position in laying/hatching sequence as covariates and biological mothers ID and foster mothers ID as crossed random factors. As with the models of egg and yolk mass, laying order and hatching order were measured as relative values by dividing the egg laying order by the clutch size and dividing the hatching order by the brood size respectively. In addition, sex was included as a fixed factor in the model of mass at 35 days post-hatch.

In all models I included all possible two-way interactions between the explanatory variables in the full model. I then simplified models by stepwise removal of non-significant terms starting with the interactions. Interaction terms are only shown when they were statistically significant. Non-significant main effects were kept in the final model only if they were part of a significant interaction. Tables include all main effects, including non-significant effects that were excluded from the final model. All tests were two-tailed and $p<0.05$ was considered statistically significant. All mean values are presented with ± standard error unless otherwise stated.

2.4 Results

2.4.1 Female Body Mass and Egg Production

Body mass was slightly lower in inbred females compared to control females (Table 2.1) but there was no statistically significant effect of inbreeding or replicate on body mass (general linear mixed model, inbreeding: t=0.35, n=64, p=0.726; replicate: t=0.573, p=0.572). However, when mass was analysed for a larger group of zebra finches (including males and females) approximately one year later, inbred females (17.34±0.27) were significantly lighter than control females (18.90± 0.30, t=2.10, n=169 (33 families), p=0.043 general linear mixed model; inbreeding depression of 8.25%). In the larger sample, inbred birds were also found to be skeletally smaller than control females (general linear mixed model of PCA of tarsus, head-bill and wing length; t=2.11, n=169 (33 families), p=0.043). Therefore, the lack of significant difference between inbred and control females may be due to a lack of statistical power.

Similar proportions of inbred females (24 out of 32) and control females (26 out of 32) successfully produced a first clutch (χ^2-test, χ_1^2=0.37, p=0.545). Median latency to lay was 10 days (range 6-43 days) and did not differ between inbred and control females, or between replicates (generalised linear models with a quasi-Poisson distribution, inbreeding status: t=1.18, n=50 (28 families), p=0.245; replicate: t=1.36, p=0.181). There was no effect of inbreeding on clutch size, but clutches were larger in the first replicate (5.0±1.20 eggs, n=24) than in the second replicate (4.1±1.14 eggs, n=26) and clutch size declined with increasing latency to lay (Table 2.2).

Of the 223 eggs that were laid, egg and yolk masses were measured for 196 eggs; the remaining 27 eggs could not be used because these measurements could not be obtained (10 eggs from 9 clutches from control females and 17 eggs from 12 clutches, including both eggs of a clutch of 2, from inbred females). That left a sample of 90 eggs laid by 23 inbred females from 15 different families and 106 eggs laid by 26 control females from 13 different families. Larger females laid larger eggs and, when statistically accounting for female body mass, egg mass was associated non-additively with inbreeding status, clutch size and laying order (Tables 2.2; Fig. 2.2a). There was an effect of inbreeding status on egg mass depending on clutch size; inbred females laid significantly smaller eggs than control females when laying small clutches, whereas egg mass did not differ between inbred and control females in large clutches (Fig. 2.2a). There was also an interaction

between clutch size and laying order; egg mass decreased significantly with laying order in small clutches ($t=2.70$, $n=88$, $p=0.009$) and showed an increase with laying order in large clutches, although not statistically significant ($t=1.83$, $n=108$, $p=0.070$). Inbred females laid eggs with smaller yolks than control females when accounting for body size and there was an interactive effect between clutch size and laying order (Table 2.2, Fig. 2.2b). Yolk mass decreased significantly with laying order in small clutches (clutches below the average clutch size of 4.5 eggs (SD=1.29); $t=2.24$, $n=88$, $p=0.029$) but there was no change in yolk mass over the laying sequence in clutches larger than the average clutch size ($t=1.60$, $n=108$, $p=0.113$).

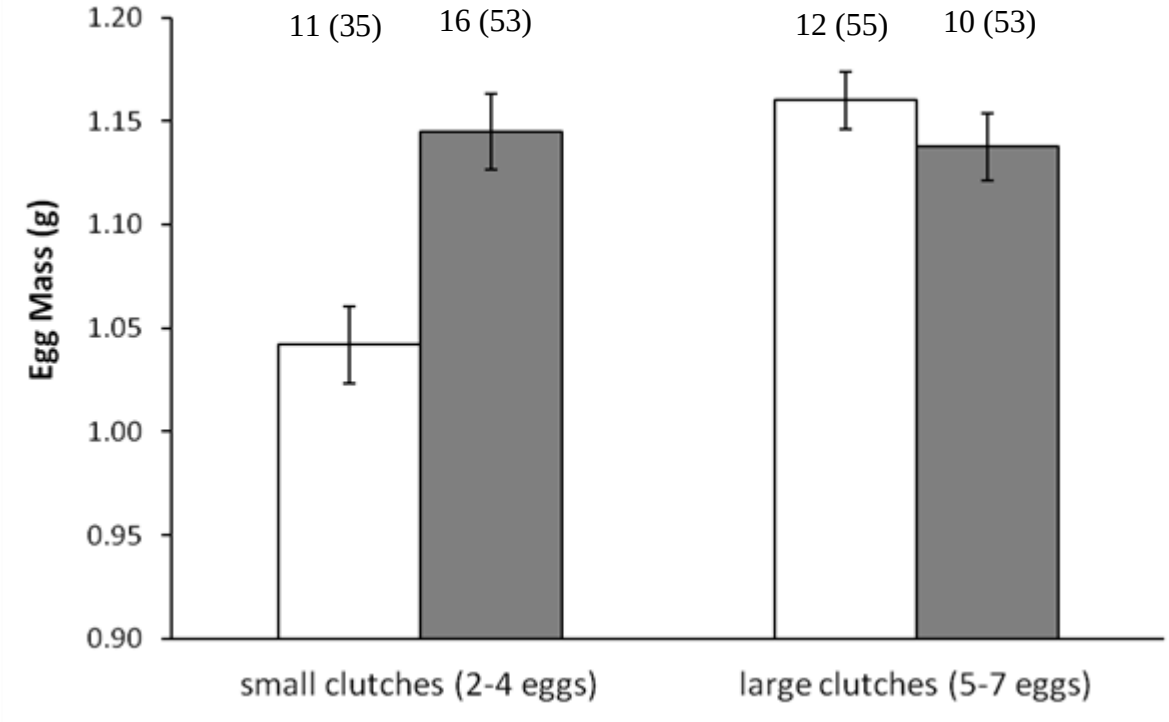
Table 2.1 Mean (±SE) traits of inbred and control zebra finch females. Inbreeding depression is the percentage change in trait value at an inbreeding coefficient *f*=0.25 and effect size of inbreeding was calculated as the mean difference in trait value divided by the pooled standard deviation of the two groups (Cohen's d). Traits showing statistically significant effects of inbreeding (or statistically significant interactions involving inbreeding) are highlighted in bold.

	Inbred	**Control**	**Inbreeding depression**	
Trait	**Mean (SE)**	**Mean (SE)**	**δ (%)**	**Cohen's d**
Body mass (g)	16.90 (0.41)	17.32 (0.43)	-2.41	-0.198
Latency to lay (days)	15.79 (2.24)	12.50 (1.56)	26.33	0.346
Clutch size	4.33 (0.26)	4.69 (0.23)	-7.65	-0.291
Egg mass (g)	**1.11 (0.01)**	**1.14 (0.01)**	**-2.38**	**-0.220**
Yolk mass (g)	**0.251 (0.003)**	**0.274 (0.004)**	**-8.30**	**-0.645**
Lysozyme concentration (μg/ml)	0.0062(0.003)	0.0060(0.003)	3.10	0.08
Lysozyme content (μg/egg)	**0.0049(0.0003)**	**0.0047(0.0002)**	**5.26**	**0.13**
Ovotransferrin concentration (μg/ml)	6886(290)	7881(382)	-12.63	-0.38
Ovotransferrin content (μg/egg)	5385(256)	6156(306)	-12.52	-0.36
Avidin concentration (μg/ml)	0.042(0.002)	0.040(0.002)	4.84	0.22
Avidin content (μg/egg)	0.033(0.002)	0.032(0.002)	1.79	0.07
Hatching success (%)	75 (13)	82 (7)	-8.93	-0.110
Post-hatching survival to 35 days (%)	**63 (9)**	**61 (9)**	**4.31**	**0.053**
Hatchling mass (g)	**0.80 (0.02)**	**0.84 (0.03)**	**-4.08**	**-0.243**
Chick mass at 35 days (g)	14.2 (0.67)	14.71 (0.45)	-3.66	-0.229

Table 2.2 General linear mixed models comparing (a) clutch size, (b) egg mass and (c) yolk mass between inbred (90 eggs laid by 23 females from 15 families) and control (106 eggs laid by 26 females from 13 families) female zebra finches, accounting for replicate, and relevant covariates (see methods). All possible two-way interactions were tested but only statistically significant interactions are shown; all other interactions were not significant (clutch size: p≥0.292; egg mass: p≥0.121; yolk mass: p≥0.062). Non-significant main effects were dropped stepwise from the final model.

Model	Explanatory Variable	t		Estimate (SE)
(a) Clutch size			p	
	Inbreeding status	0.37	0.711	
	Replicate	**4.27**	**>0.001**	**1.327 (0.311)**
	Latency to lay	**3.50**	**0.002**	**-0.948 (0.271)**
	Random effects		σ^2 (%)	
	Family		0.35	
(b) Egg Mass			p	
	Body mass	**2.74**	**0.013**	**0.019 (0.007)**
	Inbreeding	**2.97**	**0.006**	**0.288 (0.097)**
	Replicate	1.77	0.079	
	Clutch size	1.63	0.120	
	Laying order	**2.32**	**0.022**	**-0.180 (0.077)**
	Inbreeding * clutch size	**2.73**	**0.014**	**0.055 (0.020)**
	Clutch Size * laying order	**2.40**	**0.018**	**0.037 (0.015)**
	Random effects		σ2 (%)	
	Nest (nested within Family)		47.58	
	Family		47.39	
(c) Yolk Mass			p	
	Body mass	**2.65**	**0.016**	**0.006 (0.002)**
	Inbreeding	**2.39**	**0.025**	**0.022 (0.009)**
	Replicate	0.56	0.575	
	Clutch size	1.32	0.204	
	Laying order	**2.25**	**0.026**	**-0.048(0.022)**
	Clutch size * laying order	**2.25**	**0.026**	**0.010 (0.004)**
	Random effects		σ2 (%)	
	Nest (nested within Family)		13.37	
	Family		48.55	

(a)



(b)

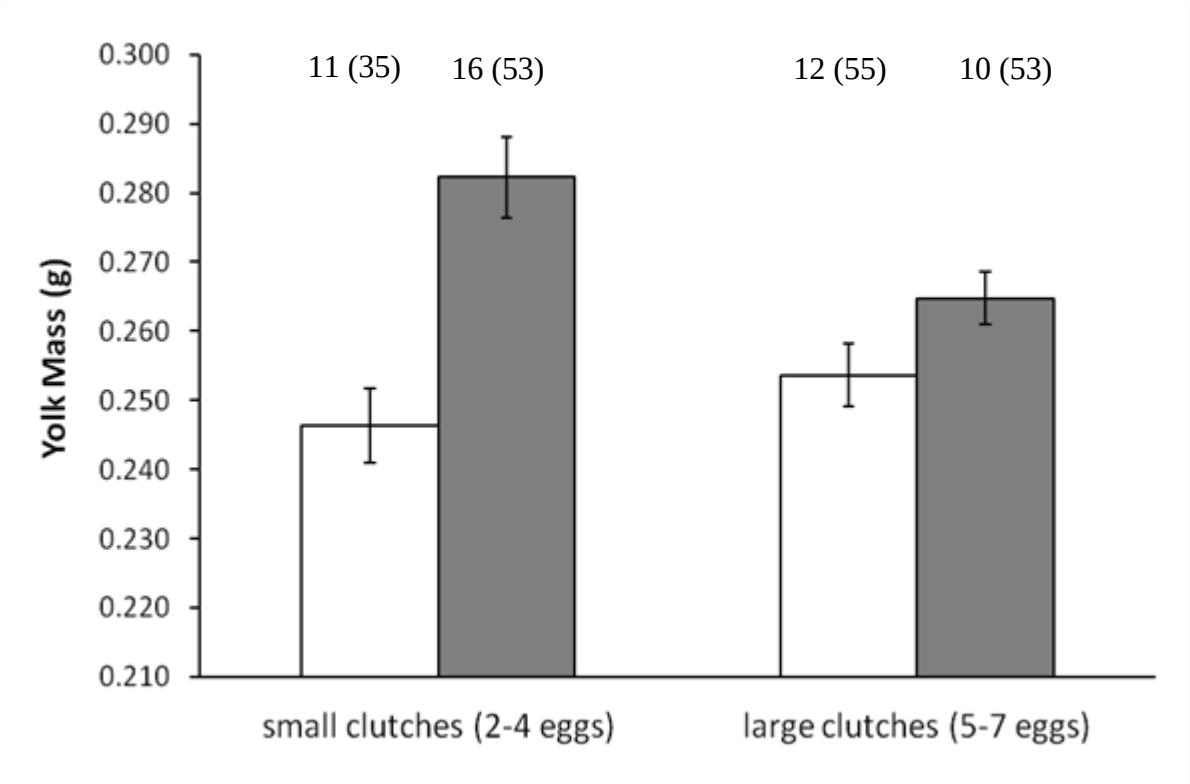


Figure 2.2 Mean (± 1SE) egg mass (a) and yolk mass (b) of inbred (open bars) and control (filled bars) females that laid small and large clutch (laid fewer or more eggs than the average clutch size, 4.53±SD1.29, n=49). The two clutch size groups are for presentation purposes only; clutch size was treated as a continuous variable in the analysis (Table 2.2). (a) In small clutches (2-4 eggs), inbred females laid significantly smaller eggs than control females (t=2.70, p=0.035) whereas egg mass did not differ between inbred and control females in large clutches (5-7 egg; t=0.14, p=0.888). (b) Inbred females always laid eggs with smaller yolks than control females (Table 2.2). The interaction between inbreeding status and clutch size was not significant (t=1.87, p=0.077). Numbers above the bars show number of nests (number of eggs).

2.4.2 Antimicrobial Protein Levels

Measures of lysozyme and ovotransferrin were not available for all eggs, as albumen was too viscous to allow accurate pipetting, or because the assay failed to yield a satisfactory standard curve and there was insufficient amount of albumen left to repeat the sample. Lysozyme was analysed for 51 eggs of 21 inbred females from 14 families and 58 eggs of 24 control females from 13 families. Lysozyme concentration was not associated with inbreeding (Tables 2.1 and 2.3; Fig. 2.3a), clutch size, laying order or replicate (Table 2.3); however, there was a marginally significant decline in lysozyme concentration with log latency to lay (Table 2.3). Total amount of lysozyme per egg was not associated with laying order, replicate, or log latency to lay, however, there was a significant interaction between inbreeding and clutch of lysozyme (Table 2.3; Fig 2.3b). Ovotransferrin was analysed for 57 eggs of 21 inbred females from 15 families and 60 eggs of 24 control females from 12 families. Ovotransferrin concentration and total quantities were lower in inbred females than in control females although the difference was not statistically significant (Tables 2.1 and 2.3; Fig. 2.3c; Fig. 2.3d), but were not associated with clutch size, laying order, replicate or log latency to lay (Table 2.3). Avidin concentration and total amounts of avidin were analysed for pooled samples of 17 inbred females from 13 families and 23 control females from 13 families. Avidin concentration was not associated with inbreeding, clutch size or replicate (Tables 2.1 and 2.3; Fig 2.3e), but avidin showed a marginally significant decline with latency to lay (Table 2.3). Total amounts of avidin were not associated with inbreeding, clutch size, replicate or log latency to lay (Table 2.1 and 2.3, Fig.2.3f).

Table 2.3 General linear mixed models comparing concentration (a, c, e) and total amounts per egg (b, d, f) of lysozyme (a and b), ovotransferrin (c and d) and avidin (e and f). All possible two-way interactions were tested but only significant interactions are shown, all other interactions were non-significant (lysozyme conc. $p \geq 0.647$; total lysozyme $p \geq 0.647$; ovotransferrin conc. $p \geq 0.127$; total ovotransferrin $p \geq 0.2446$; avidin conc. $p \geq 0.111$; total avidin $p \geq 0.251$). Non-significant main effects were dropped stepwise from the final model. Samples sizes for lysozyme were 51 eggs of 21 inbred females (14 families) and 58 eggs of 24 control females (13 families). Sample sizes for ovotransferrin were 57 eggs of 21 inbred females (15 families) and 60 eggs of 24 control females (12 families). Sample sizes for avidin were 17 inbred females (13 families) and 23 control females (13 families).

Model	Explanatory Variable	t		Estimate (SE)
(a) Lysozyme (concentration)			**p**	
	Inbreeding	0.87	0.392	
	Replicate	0.62	0.545	
	Clutch size	0.21	0.836	
	Laying order	0.08	0.951	
	Laying order squared	1.59	0.117	
	Log latency to lay	1.81	0.088	-0.742 (0.409)
	Random effects		**σ^2 (%)**	
	Nest (nested within family)		<0.00	
	Family		<0.00	
(b) Lysozyme (total per egg)			**p**	
	Inbreeding	**2.13**	**0.043**	**2.556 (1.195)**
	Replicate	1.27	0.224	
	Clutch size	**2.43**	**0.027**	**0.415 (0.171)**
	Laying order	1.43	0.158	
	Laying order squared	0.07	0.947	
	Log latency to lay	1.48	0.160	
	Inbreeding * clutch size	**2.42**	**0.028**	**-0.582 (0.241)**
	Random effects		**σ^2 (%)**	
	Nest (nested within family)		1.13	
	Family		0.00	
(c) Ovotransferrin (concentration)			**p**	
	Inbreeding	1.98	0.059	
	Replicate	0.96	0.354	
	Clutch size	1.13	0.276	
	Laying order	0.68	0.499	
	Laying order squared	1.21	0.229	
	Log latency to lay	0.37	0.713	
	Random effects		**σ^2 (%)**	
	Nest (nested within family)		0.00	
	Family		7.74	

Model	Explanatory Variable	t		Estimate (SE)
(d) Ovotransferrin (total per egg)			**p**	
	Inbreeding	1.96	0.061	5321 (326)
	Replicate	1.39	0.183	
	Clutch size	0.70	0.494	
	Laying order	0.94	0.347	
	Laying order squared	0.74	0.461	
	Log latency to lay	0.28	0.783	
	Random effects		**σ^2 (%)**	
	Nest (nested within family)		0.00	
	Family		10.47	
(e) Avidin (concentration)			**p**	
	Inbreeding	0.74	0.469	
	Replicate	1.00	0.336	
	Clutch size	0.40	0.699	
	Log latency to lay	2.09	0.057	
	Random effects		**σ^2 (%)**	
	Family		12.03	
(f) Avidin (total per egg)			**p**	
	Inbreeding	0.15	0.885	
	Replicate	1.62	0.131	
	Clutch size	0.28	0.782	
	Log latency to lay	1.76	0.101	
	Random effects		**σ^2 (%)**	
	Family		23.01	

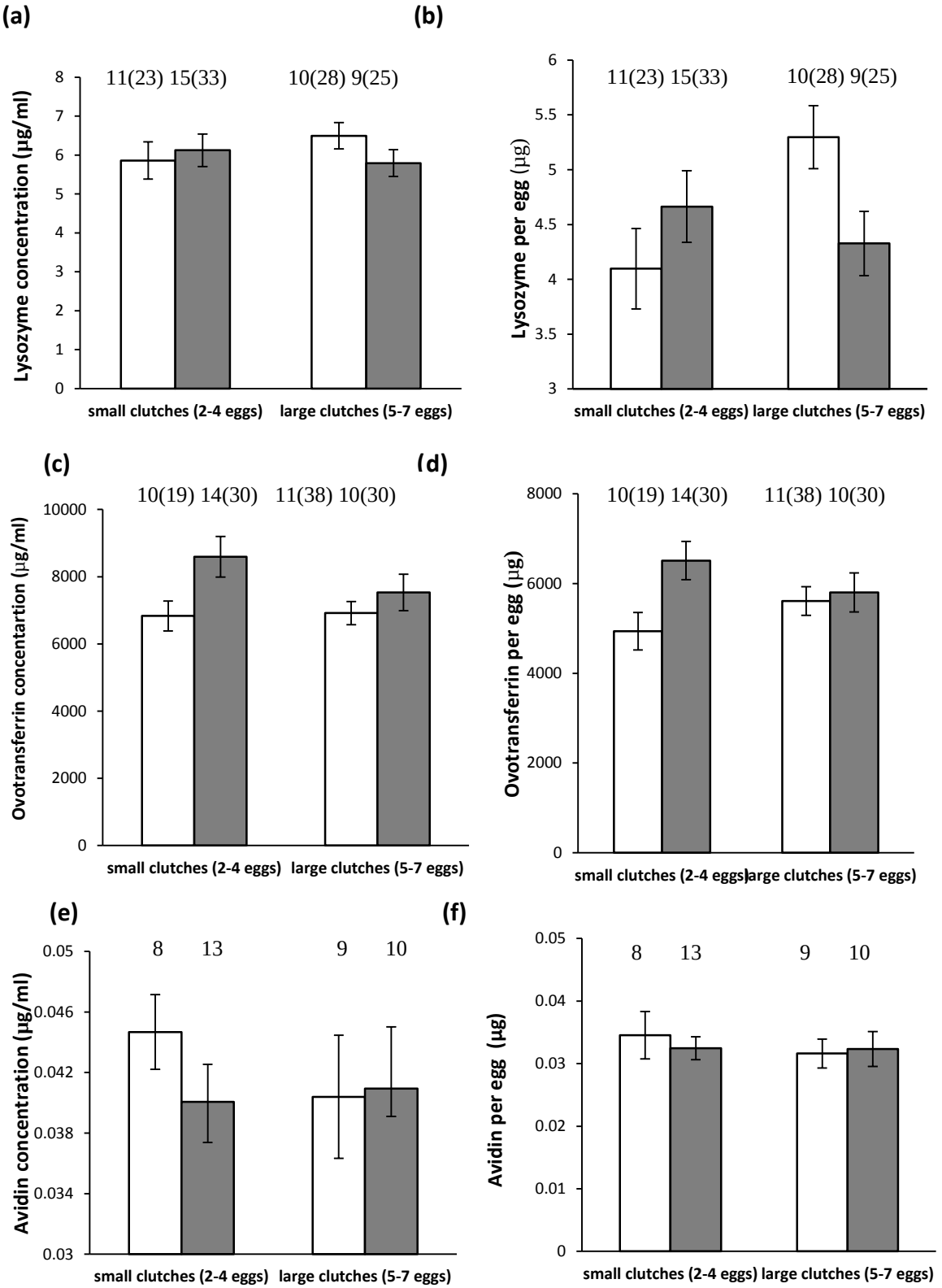


Figure 2.3 Mean (±1SE) concentration (a, c and e) and total (b, d and f) lysozyme (a and b), ovotransferrin (c and d) and avidin (e and f) of inbred (open bars) and control females (filled bars) for females that laid small and large clutches (laid fewer or more eggs than the average clutch size (4.53±SD1.29, n=49), respectively. Clutches were split into below and above average clutch size for presentation only; clutch size was included as a covariate and not a factor in the models (Table 2.3). In large clutches (5-7 eggs), total amounts of lysozyme per egg were higher in inbred females compared to control females (t=2.20, n=108, p=0.042), however, there was difference in total amounts of lysozyme per egg between inbred and control females in small clutches (t=1.00, n=88 p=0.326).

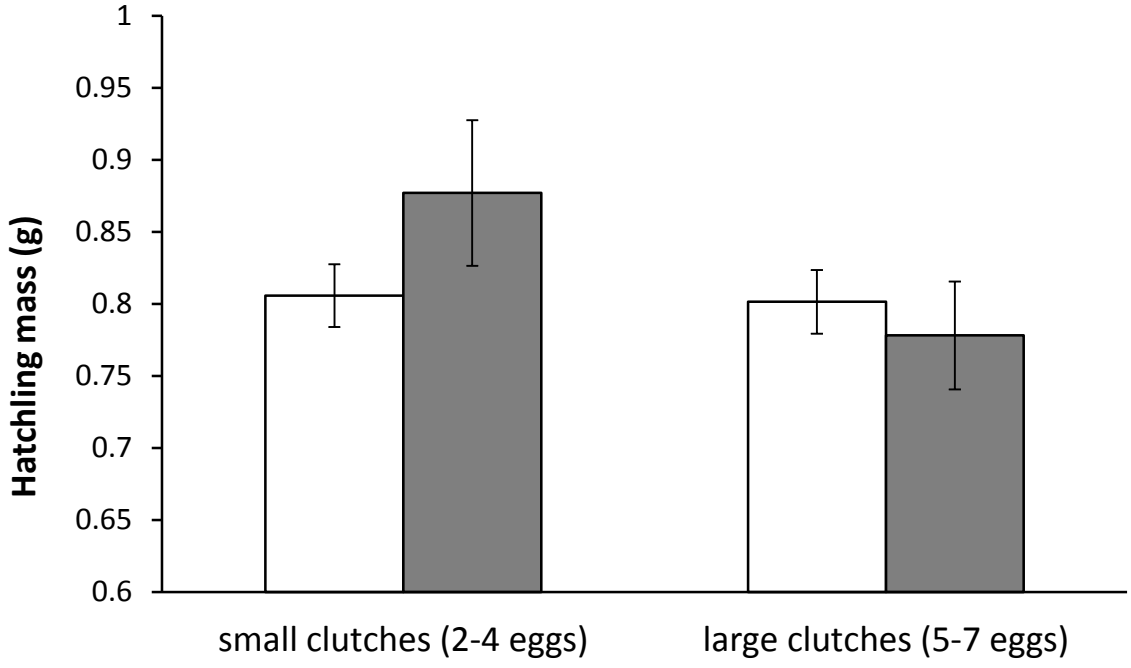
2.4.3 Offspring Viability

Mean hatching success, post-hatching survival, hatchling mass and mass at day 35 for eggs laid by inbred and control females are shown in Table 2.1 and include only offspring that were fostered to, and incubated and raised by, control mothers. Hatching success was analysed for 74 eggs originating from 15 control females and 18 inbred females and cross fostered to 17 control females. Hatchling mass and survival was analysed for 58 chicks, originating from 13 control females and, 15 inbred females fostered by 16 control females. Mass at 35 days was analysed for 32 chicks from 10 control females and 11 inbred females and cross fostered by 14 control females. Although there appeared to be a strong inbreeding depression on hatching success (Table 2.1), there was no statistically significant effect of the inbreeding status of the biological mother, replicate or clutch size on hatching success, although hatching success declined with laying order (Table 2.4). Post-hatching survival to independence (35 days of age) did not vary with replicates or hatching order but was significantly higher in females compared to male chicks (Table 2.4). Accounting for sex differences in post-hatching survival, offspring survival differed between chicks hatched from eggs laid by inbred and control females, depending on brood size (Table 2.4). Post-hatching survival declined with brood size in chicks of control mothers ($z=2.36$, $p=0.019$), but was not affected by brood size in chicks of inbred mothers ($z=0.15$, $p=0.880$). Hatchling mass was related non-additively to inbreeding, replicate and clutch size but not laying order (Table 2.4). In small clutches the chicks of inbred mothers were slightly smaller than control females, although not significantly, but in large clutches they showed the opposite pattern, although not significantly (Fig 2.4a). There was no difference in hatchling mass between chicks of inbred and control mothers in the first replicate ($t=0.85$, 37 chicks from 15 females $p=0.424$), but hatchlings from inbred mothers were significantly lighter than hatchlings from control mothers in the second replicate ($t=1.84$, 21 chicks from 13 families, $p=0.037$; Fig. 2.4b). Mass at 35 days did not vary with inbreeding, replicate, or offspring sex, but declined with increasing brood size and hatching order (Table 2.4).

Table 2.4 Results of statistical models comparing (a) Hatching success, (b) Survival to 35 days post hatch, (c) Hatchling mass and (d) Mass at 35 days post-hatch between inbred and control female zebra finches accounting for replicate, sex (in the survival to 35 days post hatch and mass at day 35 models only) and relevant covariates. All other interactions were non-significant (Hatching success p≥0.559, survival to 35 days post hatch p≥0.072, hatchling mass p≥0.173, mass at 35 days post-hatch p≥0.346). Non-significant main effects were dropped stepwise from the final model.

Model	Explanatory Variable			Estimate (SE)
(a) Hatching success		**z**	**p**	
	Replicate	0.27	0.788	
	Inbreeding	0.32	0.749	
	Clutch Size	1.03	0.304	
	Laying order	**2.05**	**0.041**	**-13.50 (6.60)**
	Random effects		σ^2 (%)	
	Nest of origin		185.56	
	Foster nest		68.21	
(b) Survival 35 days post hatch		**z**	p	
	Replicate	**2.35**	**0.019**	**3.52 (1.50)**
	Inbreeding	**2.23**	**0.026**	**9.14 (4.10)**
	Brood size	**1.41**	**0.180**	**-0.96 (0.71)**
	Hatching order	0.83	0.410	
	Sex	**2.39**	**0.017**	**2.19 (0.92)**
	Inbreeding * brood size	**2.33**	**0.020**	**2.33 (1.00)**
	Random effects		σ^2	
	Nest of origin		0.00	
	Foster nest		1.63	
(c) Hatching mass		**t**	p	
	Replicate	**0.19**	**0.201**	**0.012 (0.064)**
	Inbreeding	**1.44**	**0.146**	**0.299 (0.208)**
	Clutch Size	**2.27**	**0.020**	**0.056 (0.025)**
	Laying order	2.84	0.643	
	Inbreeding * clutch size	**2.13**	**0.024**	**0.105 (0.049)**
	Inbreeding * replicate	**2.16**	**0.022**	**-0.216 (0.10)**
	Random effects		σ^2 (%)	
	Nest of origin		44.60	
	Foster nest		0.00	
(d) Mass 35 days post hatch		**t**	p	
	Replicate	0.90	0.339	
	Inbreeding	0.12	0.999	
	Brood size	**2.63**	**0.011**	**-1.38 (0.52)**
	Hatching order	**2.18**	**0.039**	**-2.73 (1.26)**
	Sex	0.83	0.364	
	Random effects		σ^2 (%)	
	Nest of origin		46.67	
	Foster nest		8.98	

A



B

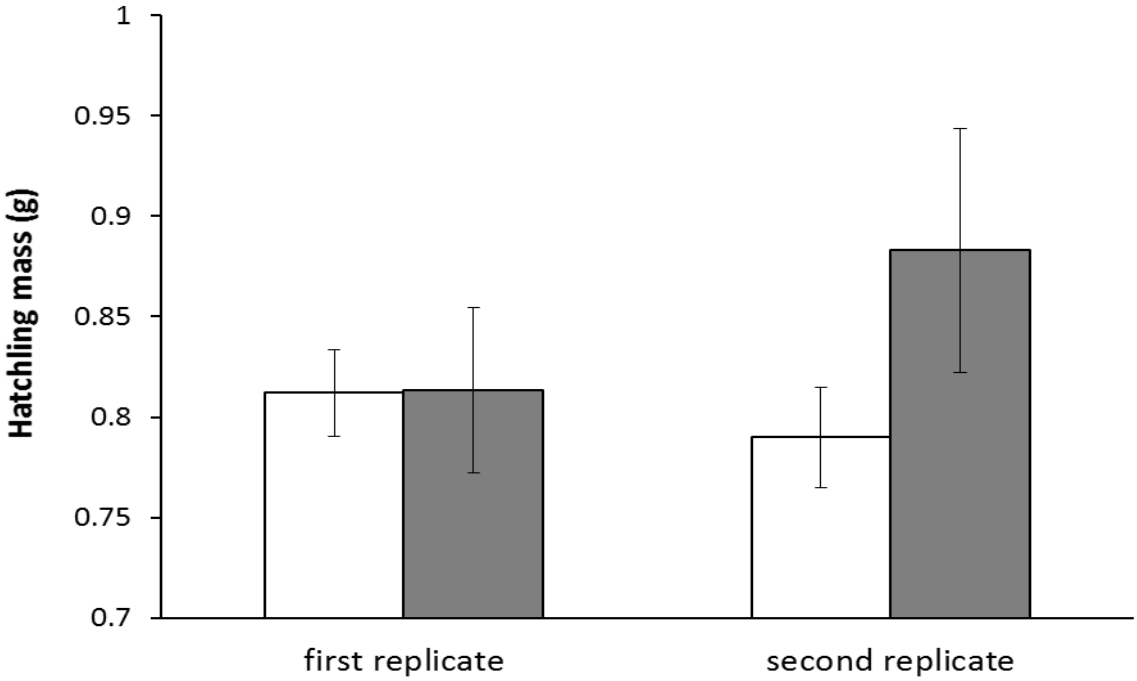


Figure 2.4: Mean (± 1SE) hatchling mass for chicks with inbred biological mothers (open bars) and control biological mothers (filled bars). There was a significant interaction between clutch size and inbreeding status of the biological mother for hatchling mass (Table 2.4). In small broods, chicks of inbred females were smaller than control females although this trend was not statistically significant (t=1.49, p=0.123). In large clutches, chicks of inbred females were slightly larger than chicks of control females although not statistically significant (t=0.34, p=0.271). There was also a significant interaction between the inbreeding status of the biological mother and replicate. In the first replicate hatchling mass did not differ between chicks of inbred and control mothers (t=0.85, p=0.424), however, chicks of inbred females were significantly smaller than chicks of control females in the second replicate (t=2.02, p=0.037).

2.5 Discussion

This study investigated, firstly, whether maternal inbreeding leads to a decline in egg production (in terms of clutch size, egg mass, yolk mass, levels of antimicrobial proteins) and, secondly whether inbreeding in the egg laying mother has a negative impact of offspring viability. While previous studies suggested effects of inbreeding on clutch size and egg volume, maternal inbreeding in captive zebra finches with pedigree information did not influence the probability to lay a clutch, clutch size, or latency to production of the first clutch, but, to my knowledge, this is the first study to show an effect of maternal inbreeding on egg quality. I found differences in egg mass and yolk mass between inbred and control females. Inbred females laid smaller eggs for their body mass compared with control females but this difference was only found in females that laid clutches of below-average size. Inbred females also produced eggs with smaller yolks than control females, independent of body mass and clutch size. Within-clutch differences in egg and yolk mass with laying sequence did not differ between clutches of inbred and control females, but instead, varied with clutch size. There was no consistent effect of inbreeding on the concentration or total amounts of antimicrobial proteins in the egg. Changes in egg quality can have important fitness consequences. Mass of hatchlings from eggs laid by inbred and control mothers (all raised by control foster parents) showed a similar pattern to that of egg mass, although hatchling mass was also affected by whether females bred in the summer (first replicate) or autumn (second replicate). Hatching success was not affected by whether eggs were laid by inbred or control mothers, but post-hatching survival (to day 35) was affected by the interactive effects of inbreeding of the biological mother and brood size.

Several studies have found correlations between the level of heterozygosity (often used as a proxy for inbreeding) and several aspects of maternal egg production, including clutch size (Foerster *et al.* 2003; Tomiuk *et al.* 2007; Ortego *et al.* 2007; García-Navas *et al.* 2009; Olano-Marin *et al.* 2011; Wetzel *et al.* 2012) and laying date (Tomiuk *et al.* 2007). However, heterozygosity is not necessarily a good indication of inbreeding (Pemberton 2004). A few studies have investigated maternal inbreeding based on pedigree information and also found that maternal inbreeding status can delay laying date (Marr *et al.* 2006) and reduce the number of eggs laid (Sittmann *et al.* 1966; Sewalem *et al.* 1999). However, in my study, neither the latency between pairing and onset of laying or clutch size was statistically significantly affected by inbreeding. Clutch size was, however, reduced by 7.7% in inbred females and latency to lay was extended by over three days on average,

around 26.3%, in inbred females, which may be considered moderate to high effects compared to inbreeding depression in other traits (DeRose & Roff 1999). It is possible that both these traits may show statistically significant effects with a larger sample size.

Inbred birds laid smaller eggs, but only in females that laid clutches of below-average size, while I found the strongest effect of inbreeding was on yolk mass, with inbred females laying eggs with smaller yolks than control females. The differences in the size of eggs and their yolk between eggs laid by inbred and control mothers were not caused by differences in body mass between inbred and control females, as body mass was statistically controlled for. An effect of inbreeding on egg size has rarely been observed in previous studies (pedigree information: Sittmann *et al.* 1966; Sewalem *et al.* 1999; multilocus heterozygosity, Wetzel *et al.* 2012), and no previous study has examined the effect of inbreeding on egg quality, although García-Navas *et al.* (2009) found a correlation between increasing homozygosity and reduced evenness of egg shell spotting in blue tits. In the same study, increased evenness of shell spotting was positively associated with the number of fledged chicks per nest.

It is not clear why inbred females producing large clutches did not show inbreeding depression in egg mass whereas females laying small clutches did. Clutch size may reflect female quality (e.g. Slagsvold and Lifjeld 1990; Christians 2002; Garamszegi *et al.* 2004) which may explain why inbred females laying clutches of above-average size did not show a reduced egg mass. Despite this, inbred females laying above-average clutch sizes laid eggs with significantly smaller yolks, and thus even the inbred females that were able to produce a large number of eggs suffered reduced yolk mass. Inbreeding depression can vary depending on circumstances (reviewed in Armbruster & Reed 2005) and in this case females laying above-average clutches appear to make up for the smaller yolk by producing larger albumens, resulting in eggs of similar size as those laid by control females. Albumen is mainly made up of water and protein and as all inbred females appear to deposit less nutrients into the yolk (protein and lipid), inbred females are most likely to increase water content of their yolk. Further studies of yolk and albumen composition of eggs laid by inbred birds could help us to better understand what nutrients are limiting egg production in inbred females.

Within-clutch patterns of egg and yolk mass across the laying sequence did not differ between inbred and control females. However, there was a significant interaction between laying sequence and clutch size for both egg mass and yolk mass. In females that laid

clutches of above-average size there were non-significant increases in egg and yolk mass across the laying sequence, whereas egg and yolk mass significantly declined with laying order in females that laid clutches of below-average size. Increased egg mass with laying sequence has been described in both captive and wild zebra finches (Griffith & Buchanan 2010; Griffith *et al.* 2011), although within-clutch patterns in egg mass can depend on environmental conditions such as the time of year (Williamson *et al.* 2008) or diet quality (Rutstein *et al.* 2004). It may be that females laying small clutches are of lower quality and/or in poorer body condition than females laying large clutches and therefore may not be able to maintain their egg production expenditure throughout the laying sequence, and as a result are forced to reduce egg mass and yolk mass. This is perhaps why females laying clutches of below-average size also show a stronger inbreeding depression in egg size than females laying clutches of above-average size.

The reason for the decline in egg size and yolk size in inbred females is not known. Birds had to work for the majority of their food as seeds were either scattered on the floor or the birds had to fly and cling to free-hanging millet sprays (Law *et al.* 2010). Foraging, particularly during an energetically and nutritionally demanding time such as egg production, may therefore be a significant challenge to the birds. Control females may be better able to cope with this challenge than inbred females and therefore able to devote more resources to egg production; this could either be due to increased foraging ability or greater metabolic scope. Inbreeding led to reductions in condition of white footed mice, *Peromyscus leucopus noveboracensis*, after reintroduction in to a natural habitat (Jiménez *et al*. 1994). Inbreeding may elevate maintenance costs compared to control birds and result in less energy that can be allocated to activities such as reproduction or sexual signalling (Ketola & Kotiaho 2009a; Ketola & Kotiaho 2009b). In line with these predictions, female zebra finches used in this study do indeed show increased resting metabolic rate for their size (chapter 4.4.1).

In addition to macronutrients (protein and lipids), the mother also transfers several micronutrients (hormones, immunoglobulins, antimicrobial proteins, egg antioxidants, RNA) to the egg that can all act as mediators of maternal effects by which mothers might adjust offspring phenotype to prevailing circumstances (Williams 2012). Other than through reduced amounts of protein and lipid resources for embryo growth and survival, embryo survival might also be affected by inadequate protection from pathogens. I have therefore hypothesised that eggs of inbred mothers not only contain fewer macronutrients but may also contain fewer antimicrobial proteins that protect the developing embryo. It

should be noted that repeatability of the antimicrobial protein measurements was lower than in two previous studies comparing eggs across species, and eggs within a wild population; lysozyme 0.79/0.85, ovotransferrin, 0.86/0.78, avidin, 0.89/0.75 (Shawkey *et al.* 2008; D'Alba *et al.* 2010). At the same time, the coefficients of variance in my zebra finch eggs were 0.35, 0.38 and 0.22 for lysozyme, ovotransferrin and avidin, respectively whereas it was 1.17, 1.33 and 8.22 for lysozyme, ovotransferrin and avidin, respectively, for wild blue tits (D'Alba *et al.* 2010). My sample of captive birds possibly represented a more homogenous sample due to them all being exposed to a relatively uniform captive environment. Since repeatability is based on the ratio of among group variance to the total variance, i.e. among group plus within group variance (Lessells & Boag 1987), a reduction in variation between groups (or individuals in our case) will cause a decline in calculated repeatability even if within-individual variance (i.e. technical error) is not increased.

To the best of my knowledge this is the first study that has specifically looked at the effects of inbreeding on antimicrobial proteins in birds' eggs. The levels of the three antimicrobial proteins in the eggs appeared to be independent of one another as they showed no significant covariance and results of the PCA showed that variance was evenly distributed over the three principle components (section 2.3.6). I also found no patterns of ovotransferrin or lysozyme allocation with laying position (this was not possible to test with avidin). Other studies have occasionally found within-clutch patterns in some antimicrobial proteins but not others (Bonisoli-Alquati *et al.* 2010; D'Alba *et al.* 2010; Shawkey *et al.* 2008), and patterns could vary with offspring sex (Bonisoli-Alquati *et al.* 2010). Differences between patterns in antimicrobial proteins could be because they are under the control of different mechanisms (D'Alba *et al.* 2010; Bonisoli-Alquati *et al.* 2010).

There was a reduction in the concentration and total amount of ovotransferrin in eggs laid by inbred females compared to control females (14.0% and 14.7%, respectively), although this difference was not statistically significant in either case. In contrast, eggs of inbred females contained larger total amounts of lysozyme in their eggs than eggs of control females, but only for females that laid clutches of above-average size. There was no difference in the amount or concentration of avidin deposited in eggs of inbred and control females. If the deposition of antimicrobial proteins is costly (Lochmiller and Deerenberg 2000; Saino *et al*. 2002 but see Shawkey *et al.* 2008; D'Alba *et al.* 2010), inbred mothers may be expected to reduce levels of antimicrobials in a similar way as they did for levels of macronutrients. Only the differences in levels of ovotransferrin would be consistent with

this hypothesis, although the differences are not statistically significant. Alternatively, since inbred females have a reduced immunity, they may have increased plasma levels of antimicrobial proteins to provide them non-specific antibacterial immunity. However, at least for lysozyme, protein is actively deposited into the egg, as the concentration in the egg can be up to 20 times higher than in maternal circulation (Saino *et al*. 2002) and there are tubular gland cells in the oviduct that actively deposit lysozyme into the albumen (Mandeles & Ducay, 1962). Thus, the elevated levels of lysozyme in eggs of inbred females is likely to be a strategy to deal with inbred animals' higher parasite loads (Coltman *et al.* 1999; Acevedo-Whitehouse *et al.* 2009 Hawley *et al.* 2005; Ross-Gillespie *et al.* 2007), and increased deposition of antimicrobials may compensate for increased transmission rate of pathogens to the offspring. It had also been hypothesised that birds may employ behavioural strategies to control bacterial growth, for example, through incubation behaviour (Shawkey *et al.* 2009). As inbred females incubate less than control females (chapter 3.4), they may therefore need to compensate for this by actively increasing lysozyme deposition into the albumen.

The observed effect of maternal inbreeding on egg and yolk mass could have important implications for offspring viability and mass. A large meta-analysis across birds by Krist (2011) found that egg mass predicted offspring body mass in birds, although the strength of this relationship diminished with nestling age. Egg mass has also been found to influence hatching success and post-hatching survival (reviewed in Krist 2011). Smaller eggs of inbred females may therefore at least partially explain the observed declines in survival of the offspring of inbred mothers (e.g. van Noordwijk & Scharloo 1981; Keller 1998; Cordero *et al.* 2004; Marr *et al.* 2006). In captive zebra finches, although I found an 8.9% decline in hatching success with inbreeding in the biological mother in eggs incubated by control foster mothers, this was not statistically significant. However, there was an interactive effect of inbreeding status of the biological mother and brood size for survival of offspring from hatching to 35 days when raised by control foster parents. Overall, offspring survival was 4.3% higher in chicks from control biological mothers compared to chicks from inbred biological mothers (Table 2.1). The survival of chicks of inbred biological mothers was not influenced by brood size whereas survival significantly declined with brood size in chicks of control mothers. This resulted in chicks of control mothers having higher survival than chicks of inbred mothers in small broods, but lower survival than chicks of inbred mothers in large broods. This may suggest that chicks which have been poorly provisioned at the egg stage (inbred females tended to lay smaller eggs with smaller yolks) actually perform better when conditions are poor (i.e. increased sibling

competition). Similar patterns have previously been predicted under life-history theory where individuals are predicted to perform best when environmental conditions match those that their phenotype has been "programmed" for, i.e. individuals that have poor conditions early in life performed best when conditions in later life are poor, whereas individuals that experience superior early conditions perform best when conditions in later life are good (Monaghan 2008).

Hatchling mass also differed between offspring from inbred and control biological mothers, but this depended on clutch size and replicate. Hatchling mass was not influenced by the inbreeding status of the biological mother in the first replicate, whereas in the second replicate hatchling mass was significantly higher among chicks of control biological mothers compared to inbred mothers. Hatchling mass also was larger in offspring from control mothers than from inbred mothers in females that laid below-average clutches only, but not in females laying clutches of above-average clutch size. Inbreeding did not influence the mass of 35 day old chicks (i.e. the age of independence). Clutch size also differed between females bred in the two replicates which could have been caused by seasonal effects; the first replicate was carried out in the summer and the second in the autumn. There is evidence that zebra finches, even under constant laboratory conditions, can show seasonal patterns in reproductive effort (Zann *et al.* 1995; Perfito *et al.* 2007;.Williamson *et al.* 2008).

This study has provided the first evidence that maternal inbreeding can lead to a decline in egg quality. This finding could have important implications for other studies of inbreeding depression as both the size of eggs as well as egg composition can affect offspring viability. This finding may therefore help to explain at least partially why both hatching success and post-hatching survival is often reduced in offspring of inbred parents. However, the cause of lower deposition of resources in inbred mothers remains an open question.

3 The Effect of Maternal Inbreeding on Incubation Behaviour and Embryo Viability

3.1 Abstract

Increased embryo mortality is the most commonly cited cause of reduced fitness in inbred mothers and may be the result of reduced parental investment. In this chapter I investigated the effect of maternal inbreeding on the incubation behaviour of female zebra finches and the effect of inbreeding in the incubating foster mother on offspring viability. To achieve this I compared incubation attentiveness of inbred female zebra finches *Taeniopygia guttata* (derived from full-sibling mating) with that of control females (derived from unrelated parents). I found a substantial inbreeding depression of 17% in incubation attentiveness, showing that inbreeding can significantly influence behavioural phenotype. Despite a reduction in the amount of time inbred females spent incubating, their partners were able to compensate for the reduced incubation attentiveness. Incubation temperature did not differ between inbred and control females. For control eggs that were incubated by either inbred or control females, there was no significant difference in hatching success or hatching mass. However, under natural conditions, more substantial effects of maternal inbreeding on embryo viability may be expected, and could provide an explanation for the consistent finding of decreased hatching success with increasing maternal inbreeding in birds.

3.2 Introduction

Inbreeding - mating between relatives - potentially leads to deleterious effects collectively termed inbreeding depression. Inbreeding depression can be biologically significant under natural conditions and is an important consideration in evolutionary and conservation biology (Crnokrak & Roff 1999; Keller & Waller 2002). Severe inbreeding can ultimately lead to the extinction of small isolated populations (Saccheri *et al.* 1998). Inbreeding depression is thought to occur mainly due to the unmasking of rare deleterious alleles, but reduced heterozygous advantage may also contribute (Charlesworth & Willis 2009). Inbreeding increases genetic homozygosity; reduced fitness in inbred lineages can be due to the level of homozygosity in the offspring (offspring inbreeding) and/or the parents (parental inbreeding) (Keller & Waller 2002). The mechanism of how parental inbreeding adversely impacts on the reproductive success of inbred animals is unclear. It has been suggested that the level of inbreeding in the parent may act through its adverse impacts on the level of parental care they may provide, so that parental inbreeding can be considered a maternal effect (Richardson *et al.* 2004), but the effect of inbreeding on parental care has received little attention thus far.

A consistently reported deleterious effect of parental inbreeding is the reduced survival of embryos from an inbred female, even if they are paired with an unrelated mate so that the offsprings' heterozygosity is not reduced (e.g. Sittmann *et al.* 1966; van Noordwijk & Scharloo 1981; Pulkkinen *et al.* 1998; Su *et al*. 1996; Margulis & Altmann 1997; Moura *et al*. 2000; Keller 1998; Cordero *et al*. 2004; Marr *et al.* 2006; Farkas *et al.* 2007). The underlying causes of maternally-mediated reduced viability in embryos of inbred mothers have not been determined, but could include reduced egg quality (due to reductions in provisioning to the egg), reduced parental care in supporting development, or a combination of the two (Richardson *et al.* 2004). In birds, there is evidence that inbred females may produce smaller eggs compared to control females (Sittmann *et al*. 1966; Sewalem *et al.* 1999) and offspring from smaller eggs can have a reduced fitness (Williams 1994; Krist 2011).

Avian parents must also actively maintain favourable conditions for optimal embryo development (Webb 1987). Maintaining eggs at favourable incubation conditions can be expensive both in terms of energy and time (Tinbergen & Williams 2002). Incubation expenditure can be influenced by the parent's energy balance, body condition and food

availability (Bryan & Bryant 1999; Eikenaar *et al.* 2003; Gorman & Nager 2003). Inbred individuals may be in poorer condition (Jiménez *et al.* 1994; Knaepkens *et al.* 2002) or have less energy available to spend on non-self-maintenance activities (Ketola & Kotiaho 2009a) compared to control individuals, and this could result in a lower incubation attentiveness or incubation temperature. Low parental incubation expenditure can result in slower embryo development, teratogenesis, increased bacterial growth in the egg and even mortality (Webb 1987; Tinbergen & Williams 2002; Cook *et al.* 2005). The effects of parental inbreeding on parental care such as incubation expenditure have, however, have not been studied.

Parental care could be reduced in inbred parents and may contribute to the commonly observed inbreeding depression in embryo survival, but this has been little studied. In this study, I compared incubation attentiveness and incubation temperature of inbred and control zebra finch, *Taeniopygia guttata*, females in a captive population. In zebra finches both parents participate in incubation attentiveness throughout the day time, and so the male may potentially compensate for a reduction in incubation attentiveness by the female (Zann & Rossetto 1991). I hypothesised that inbred females would spend less time incubating eggs (lower incubation attentiveness) and/or maintain eggs at a lower temperature compared to control females and this may have a concomitant effect on offspring viability. I investigated the following questions;

- Does maternal inbreeding result in a reduction in the incubation attentiveness of females?

- If maternal inbreeding does lead to a reduction in female incubation attentiveness, how does this affect the total incubation attentiveness of the clutch; i.e. do males perform compensation behaviour?

- Does maternal inbreeding lead to a reduction in the mean incubation temperature of females?

- Does inbreeding in the incubating foster mother lead to a reduction in embryo viability in terms of growth and survival?

3.3 Materials and Methods

3.3.1 Generation of Inbred and Control Lines

The study was carried out on captive zebra finches held at the University of Glasgow. An overview of the experimental design can be seen in Fig 1.1, section 1.11 and appendix one. In the winter 2008/09 I bred inbred and control zebra finch females from my stock of several hundred individuals with known pedigree since 2006; this stock is regularly replenished with birds from other populations (e.g. pet shops) in order to maintain genetic diversity. Inbreeding in captive zebra finches has been shown to be low and similar to that found in many wild avian populations (Forstmeier *et al.* 2007). To obtain inbred birds, I paired full-sibling brothers and sisters; control birds were created by pairing males and females from my stock population that did not share any grandparents and supplemented with ten females brought into the stock just prior to this experiment that were assumed to be unrelated to any stock bird. I paired up 19 brother-sister pairs and 21 pairs of unrelated birds pairs to produce the inbred and control females used in this study. Control and inbred birds were thus bred at the same time and under identical conditions and the proportion of offspring that were derived from first, second or third broods that their parents produced were similar between the two groups (Fisher's exact test: p=0.550). See chapter 2.3.1 for more details.

3.3.2 Pairing of Inbred and Control Females

The daughters of the brother-sister pairs then became the inbred females (inbreeding coefficient f=0.25), while those from the unrelated pairs became the control birds (f=0, assuming no inbreeding in the stock population). Because these experimental birds were produced over an extended period and I wanted them to breed at a similar age, the experimental breeding rounds were conducted at two time points (July 2009 and October 2009), hereafter referred to as the first and second replicate, respectively. Based on a median inbreeding depression of 11.8% for life-history traits (DeRose & Roff 1999) and the observed distribution of incubation attentiveness of females in my population (mean 62%, SD 9%, Hill *et al.* 2012) I calculated an expected Cohen's effect size of 0.82 (Nakagawa & Cuthill 2007). For this effect size, a sample size of 57 would give a statistical power of 85% at p=0.05 and therefore I decided to pair up 16 inbred and 16 control females in each of the two replicate breeding rounds (giving a total of 64 experimental females) in order to balance statistical power with logistical and welfare considerations. Each female bred in just one of the replicate breeding rounds. The age of

pairing of females and their partners did not differ between inbred and control groups (chapter 2.3.2). Each female was paired with an unrelated male with whom they did not share any grandparents. The two replicate breeding rounds were carried out at similar room temperatures (first replicate: 24.1±1.83 ºC (mean±se); second replicate: 23.2±1.13 ºC), but the effective photoperiod was likely to differ due seasonal effects. While breeding, birds received *ad libitum* water and ca. 8g soaked seed scattered on the floor of the cage as well as *ad libitum* millet seed the birds had to pick from a spray hanging from the cage ceiling (Law *et al.* 2010) and the same breeding diet supplements as above were also given but once a week only (chapter 2.3.2).

Eggs of the first clutch of each pair laid were removed before the onset of incubation for another part of the experiment (chapter 2.3.4; Fig. 1.1, section 1.11) and birds were allowed to lay a replacement clutch. During laying, nests were checked every day and laying position was marked on each freshly laid egg with a permanent marker. Incubation attentiveness and temperature were recorded for the replacement clutches (see 3.3.3 and 3.3.4). In order to allow us to separate the effects of parental incubation performance on embryo growth and survival from possible effects of parental inbreeding on egg production (Sittmann *et al.* 1966; Sewalem *et al.* 1999; chapter two), I cross-fostered eggs amongst nests of control and inbred females before the commencement of incubation (chapter 2.3.3).

3.3.3 Assessment of Incubation Attentiveness

I recorded the incubation attentiveness as the time spent on the eggs using small infrared-sensitive cameras inside the nest box (Hill *et al*., 2011). Recordings of incubation behaviour of 120 minutes each on two different days were made for 15 control females (derived from 10 different families) and 14 inbred females (from 9 different families). Recordings were made 5±1 days and 7±1 days after clutch completion for the first and second observation, respectively; this timing did not differ between inbred and control pairs (Mann-Whitney, $W \geq 178$, $p \geq 0.154$). Observations of incubation attentiveness were made between 9am and 5pm and the time did not differ between inbred and control pairs in either the first or second observation (Mann-Whitney, $W \geq 223$ $p \geq 0.585$). The nest camera was connected to a screen where up to four nests could be observed simultaneously and all observations were stored digitally. Recording of behaviour commenced after 15 minutes after camera placement, by which time at least one parent had always resumed incubation. A bird was only recorded as incubating if they were sitting on the eggs, not if they were

merely present in the nest box. I used instantaneous scan sampling at one minute intervals, whether females incubated or not, and calculated incubation attentiveness as the number of minutes spent incubating out of a possible 120 minutes. I validated this approach using data from a previous study where incubation attentiveness was estimated from continuous observation records (Gorman *et al.* 2005a); this data included records from 12 zebra finch nests where incubation attentiveness of individual females ranged from 9.6% to 92.8%. By re-sampling the continuous records at one minute intervals (Martin & Bateson 2007), I found that attentiveness estimates calculated from scan samples every minute correlated highly with incubation attentiveness derived from continuous recordings ($r=0.99$, $n=12$, $p<0.001$) and the incubation attentiveness calculated from the two methods did not differ (mean difference 0.4±0.35%; $t=1.03$, $n=12$, $p=0.323$). Female incubation attentiveness recorded for the same female on the two observation days was highly correlated ($r=0.81$, $n=29$, $p<0.001$). I also recorded the total incubation attentiveness of the pair, i.e. the proportion of time that the eggs were incubated by either parent.

3.3.4 Assessment of Incubation Temperature

I recorded incubation temperature as the temperature of one dummy egg made of Fimo clay (Staedtler, Nürnberg, Germany), which has the same thermal properties as a zebra finch egg (Gorman *et al.* 2005a). A thermistor (Tinytag^{TM} PB-5005-0M6, Gemini Data Loggers, Chichester, UK; reading resolution ± 0.05°C) was placed in the centre of the dummy egg and connected to a data logger (Tiny Tag, Tinytalk data logger, Gemini Data Loggers, Chichester, UK; temperature recorded every 2 seconds) via a thin cable that was hidden among the nest material. Before use, data loggers were calibrated in a temperature-controlled cabinet. For the measurement of incubation temperature, the dummy egg containing the thermistor replaced one egg of the clutch (which was placed in an incubator during the period of temperature recording) so that parents still incubated the correct number of eggs. At the end of recording the dummy egg was removed and the real egg returned to the nest. One measurement of mean temperature was made per female during the middle part of the day (around 12 noon) between 3 and 8 days after clutch completion and the timing of this measurement did not differ between control and inbred females, with respect either to day of incubation ($W=104.5$, $n=19$, $p=0.744$) or time of day, $W=77.5$, $p=0.327$). While recording temperature, female incubation attentiveness was observed through the nest camera. Temperature recordings were only taken when the female incubated alone. From the time the dummy egg was placed in the nest, it took up to 15 minutes of incubation for the temperature to reach an asymptote (determined by visual

inspection of the graph of temperature against time). I then calculated a mean steady-state incubation temperature at the asymptote for 30 minutes or until the female ended her incubation bout, but at least 15 minutes of steady-state incubation was recorded for each bird. Incubation temperatures were obtained for 9 inbred (derived from 7 different families) and 10 control females (derived from 8 different families). The recording duration of steady-state incubation temperature averaged 28.2 (±SD=3.67) minutes (n=19 females) and did not differ between inbred and control groups (Mann-Whitney, W=99, p=0.962).

3.3.5 Hatching Success and Embryo Growth

I compared hatching success and hatching mass between fostered eggs laid by control females only (to standardise egg quality with respect to parental inbreeding) and incubated either by pairs containing an inbred or control female. Nests were checked daily for hatching and upon hatching chicks were weighed to the nearest 0.001g. Any eggs that were destroyed by the parents or failed to hatch after 18 days of incubation were removed from the nest. Since eggs were cross-fostered among nests at the start of incubation, any differences in hatching success and hatching mass between eggs incubated by inbred and control females should be caused by difference in the incubation environment provided by the foster pair.

3.3.6 Statistical Analysis

I calculated inbreeding depression in two ways. First, I calculated the coefficient of inbreeding depression (δ) as the percentage change in trait value between control and inbred females for a change in inbreeding coefficient of f=0.25. I also calculated the effect size (Cohen's d) which gives a standardised measure of inbreeding depression (at f=0.25) that can be more readily compared across different traits (chapter 2.3.6).

Data were first checked for meeting the assumptions of parametric statistics, i.e. that residuals were normally distributed and showed homogeneity of variance. If assumptions were not met the data were either transformed in order to meet these assumptions or, if this was not possible, alternative error distributions using generalised linear models (where data were over or under dispersed) or non-parametric tests were used. Contingency tables were analysed using χ^2-tests where expected values were >5 and Fisher's exact tests where expected values were <5 (Bailey 1995).

The effect of inbreeding on clutch size was analysed using a general linear mixed model including inbreeding status, replicate and brood number (whether the female originated from either the first brood their parents produced or a subsequent brood) as fixed factors and family of origin as a random factor. Female incubation attentiveness (transformed to the power of 1.5 to obtain a normal distribution) was analysed using a general mixed model including inbreeding status of the female, replicate and brood number as fixed factors, clutch size (day 0 is the day of clutch completion) and day of incubation as covariates; since some families were represented by two or three sisters, identity family was included as random factors in the *nlme* package of R2.12.1 (R Core Development Team 2008) and female ID was also included as a random factor (nested with family) as there were two observations per female. Clutch size was included in the model because larger clutches are energetically more expensive to incubate (Biebach 1984; de Heij *et al*. 2007). Incubation day was also included as a covariate, since patterns of incubation attentiveness varies over the incubation period in this species (Gorman & Nager 2003).

Due to the smaller sample sizes for incubation temperature (one record for each of 19 females), I first explored whether incubation temperature varied with replicate, brood number, clutch size and day of incubation using univariate general linear models (GLMs), with family as a random factor. Incubation temperature did not differ between replicates and clutch size, however incubation temperature declined with increasing day of incubation. There was also a slight difference in incubation temperature between females from first and subsequent broods, although not statistically significant (univariate GLMs, replicate: $t=0.35$, $n=19$, $p=0.731$; clutch size: $t=0.54$, $n=19$, $p=0.598$; brood number $t=1.84$, $n=19$, $p=0.084$; incubation day: $t=4.48$, $n=19$, $p<0.001$). Hence when analysing for an effect of inbreeding on incubation temperature I only added day of incubation as covariate, whereas inbreeding status and brood number as fixed factors. It was not possible to transform total incubation attentiveness to meet the assumption of normality due extreme right skewed in the data, with the majority (24/30) of pairs having 100% total incubation attentiveness. Therefore to analyse the difference in total incubation attentiveness between pairs with inbred and control females, total incubation attentiveness of a pair was pooled across all four hours of observation (i.e. both of the two-hour observations) and compared between inbred and control pairs using a Mann-Whitney test.

I analysed hatching success and mass using mixed models including inbreeding status of the foster mother (all eggs were laid by control females) and replicate as fixed factors, clutch size and relative laying position as covariates, and the identities of biological and

foster mothers as crossed random factors. Relative laying position was included because hatching mass has been found to vary with laying position in zebra finches (Gorman *et al.* 2005b). Hatching success was analysed with a generalised linear mixed model with a binary response variable for each individual egg (0=unhatched, 1=hatched) using the *lmer* function in the package *lme4* of R2.12.1. Hatching mass was analysed using a general linear model using the *lmer* function in the package *lme4* of R2.12.1. Brood number was not included in models of chick mass and survival as including all of the variables in this model led to false convergences when running the models. Further analysis showed that the effect of brood caused false convergences in some of the models even in univarate analysis (i.e. when it was the only explanatory variable).

Because replicate and brood number are correlated, given that the majority of females (12/13) in the first replicate were from first broods while the majority of females in the second replicate (11/15) were from the second or third broods (see chapter 2.3.2 for more details), there is potentially a multi-collinearity between the effects replicate and brood number. I therefore tested the variance inflation factor (VIF) in any final models that included both replicate and brood number using the package *car* of R2.11.1. In all cases VIF $\leq$ 1.94 whereas only VIF > 4 are regarded as a sign of severe collinearity (reviewed in O'Brien 2007) and so collinearity is unlikely to be a problem in this study.

In all full models I included all possible two-way interactions. I then simplified full models using a backward stepwise elimination of non-significant variables starting with the least significant interaction term and then non-significant main effects. Tables show all main effects (p-values of non-significant terms corresponds to the last step they were included in the model) and only statistically significant interaction terms. All tests are two-tailed and $p<0.05$ is considered to be significant. Unless otherwise stated, reported values are mean±SE.

3.4 Results

Of the 32 inbred and 32 control females that were paired up, 24 inbred and 26 control females produced a first clutch (used in another part of the experiment; Chapter 2.3.4) and, of these, 14 inbred females and 15 control females produced and incubated a second clutch. Neither the likelihood to produce a first clutch (χ^2-test, χ^2=0.37 p=0.543) nor the likelihood to produce and incubate a second clutch (χ^2-test, χ^2=0.06, p=0.806) differed between inbred and control females. Clutch size showed no inbreeding depression (Table 3.1) and did not differ between inbred and control females (Table 3.2a), but clutches were significantly larger in the second replicate (4.9±0.25 eggs, n=15) compared to the first replicate (3.9±0.27 eggs, n=14 Table 3.2a).

Inbred females had on average a 16.9% lower incubation attentiveness than control females (Table 3.1), an effect which was similar in the two replicates (Fig. 3.1). However, female incubation attentiveness was significantly higher in the second replicate compared to the first replicate (Table 3.2b), and females originating from first broods had a higher incubation attentiveness than females originating from subsequent broods, so that after controlling for these effects the percentage decrease in incubation attentiveness due to inbreeding rose to 30.9% (Table 3.2b). The mean total incubation attentiveness by both parents together was over 99% for both inbred and control groups (Table 3.1) and did not differ between inbred and control pairs (Mann-Whitney test, W=180, p=0.198). Incubation temperature was similar in inbred and control females (Table 3.1) but this decreased throughout the incubation period (Fig. 3.2; Table 3.2c).

Hatching success of control eggs was 8.5% lower if they had been incubated by inbred mothers rather than control mothers (Table 3.1), however, this difference was not statistically significant (Table 3.3a). Hatching success was also unaffected by replicate, clutch size and relative laying position (Table 3.3a). Hatching mass of offspring from eggs incubated by inbred and control females were similar (Table 3.1). While there was no effect of inbreeding; replicate, clutch size and laying position affected hatching mass although in a complex pattern (Table 3.3b). There was an interaction between replicate and laying position, with hatching mass increasing with laying position in the first replicate (t=2.24; p=0.031, n=14) but not the second replicate. Hatching mass was also related to clutch size depending on replicate, no relationship between clutch size and hatching mass was observed in the first replicate (t=0.04, p=0.559, n=13), however, hatching mass increased with clutch size in the second replicate (t=2.44, p=0.018, n=12).

Table 3.1 Mean (±SE) traits of inbred females originating from full sibling pairings (inbreeding coefficient=0.25, n=14) and control females originating from pairings of unrelated partners (inbreeding coefficient=0, n=15). Inbreeding depression was calculated as both the coefficient of inbreeding depression δ x 100 (%) and also as Cohen's d effect size, calculated as the mean difference in trait value divided by the pooled standard deviation of the two groups (see section 3.3.6).

Trait	**Inbred**	**Control**	**Inbreeding depression**	
	Mean (SE)	**Mean (SE)**	**δ (%)**	**Cohen's d**
Clutch size	4.4±0.33 eggs	4.2±0.22 eggs	5.48	0.23
Females attentiveness	54±5 %	65±4 %	-16.9	-0.64
Total attentiveness	99.1±2 %	99.6±2 %	-0.50	-0.27
Female incubation temperature	36.42±0.24 °C	36.03±0.29 °C	1.08	0.47
Hatching success	76±10 %	83±9 %	-8.52	-0.19
Hatching mass	0.88±0.03 g	0.86±0.03 g	2.33	0.11

Table 3.2 General linear mixed models of (a) clutch size, (b) female incubation attentiveness (transformed to the power of 1.5) and (c) female incubation temperature. Explanatory variables were replicate (first and second), brood number (first vs. subsequent broods of their parents) and day of incubation and clutch size as covariates for the two incubation traits. The table shows all main effects that were tested (statistically significant main effects are shown in bold) but only significant ($p<0.05$) interaction terms. Parameter estimates (and associated standard errors) were derived from the final models. In all models the identity of the parents from which the tested females derived (family) was included as a random factor. Additionally, in the model of female incubation attentiveness, where there were two observations for each female, female identity was also added as a random factor nested within family.

	Variable			**Estimate (SE)**
(a) Clutch size		**t**	**p**	
	Inbreeding	0.15	0.881	
	Replicate	**2.99**	**0.015**	**1.06 (0.36)**
	Brood number	0.24	0.815	
	Random effects		**σ^2 (%)**	
	Family		1.7	
(b) Female incubation attentiveness		**t**	**p**	
	Inbreeding	**2.72**	**0.015**	**-30.9% (15.9)**
	Replicate	**3.32**	**0.011**	**41.1% (15.9)**
	Brood number	**2.30**	**0.050**	**-31.7% (18.2)**
	Day of incubation	0.57	0.571	
	Random effect		σ^2 (%)	
	Female identity		9.0	
	Family		13.7	
(c) Incubation temperature		**t**	**p**	
	Inbreeding	0.70	0.401	
	Day of incubation	**4.39**	**0.022**	**-0.43 °C (0.10)**
	Brood number	0.97	0.349	
	Random effect		**σ^2 (%)**	
	Family		36.6	

Table 3.3 (a) Generalised linear mixed model of hatching success and **(b)** general linear mixed model of hatching mass of offspring from eggs produced by control biological mothers that were fostered to inbred and control mothers at the start of incubation. Explanatory variables were: replicate (first and second), clutch size and laying position (relative to the number of eggs laid) as covariates. Identity of the biological mother (n=15 different families) and identity of the foster mother (n=29) were included as crossed random factors in both models. Table shows all main effects that were tested (significant variables in bold) and statistically significant ($p<0.05$) interaction terms. P values for the hatching mass analysis were obtained by comparing models using the ANOVA function in R2.12.1.

	Variable			**Estimate (SE)**
(a) Hatching success		**z**	**p**	
	Inbreeding status	0.19	0.598	
	Replicate	0.04	0.972	
	Clutch size	0.60	0.548	
	Laying position	0.57	0.569	
	Random effects		**σ^2 (%)**	
	Foster nest		46.9	
	Nest of origin		8.4	
(b) Hatching mass		**t**	**p**	
	Inbreeding status	0.33	0.782	
	Replicate	0.93	0.663	0.27 (0.29)
	Clutch size	2.44	0.133	0.13 (0.05)
	Laying position	1.82	0.218	-0.14 (0.11)
	Replicate * Clutch size	**1.82**	**0.043**	**0.12 (0.06)**
	Replicate * Laying position	**2.42**	**0.013**	**0.32 (0.13)**
	Random effects		**σ^2 (%)**	
	Foster nest		39.6	
	Nest of origin		44.8	

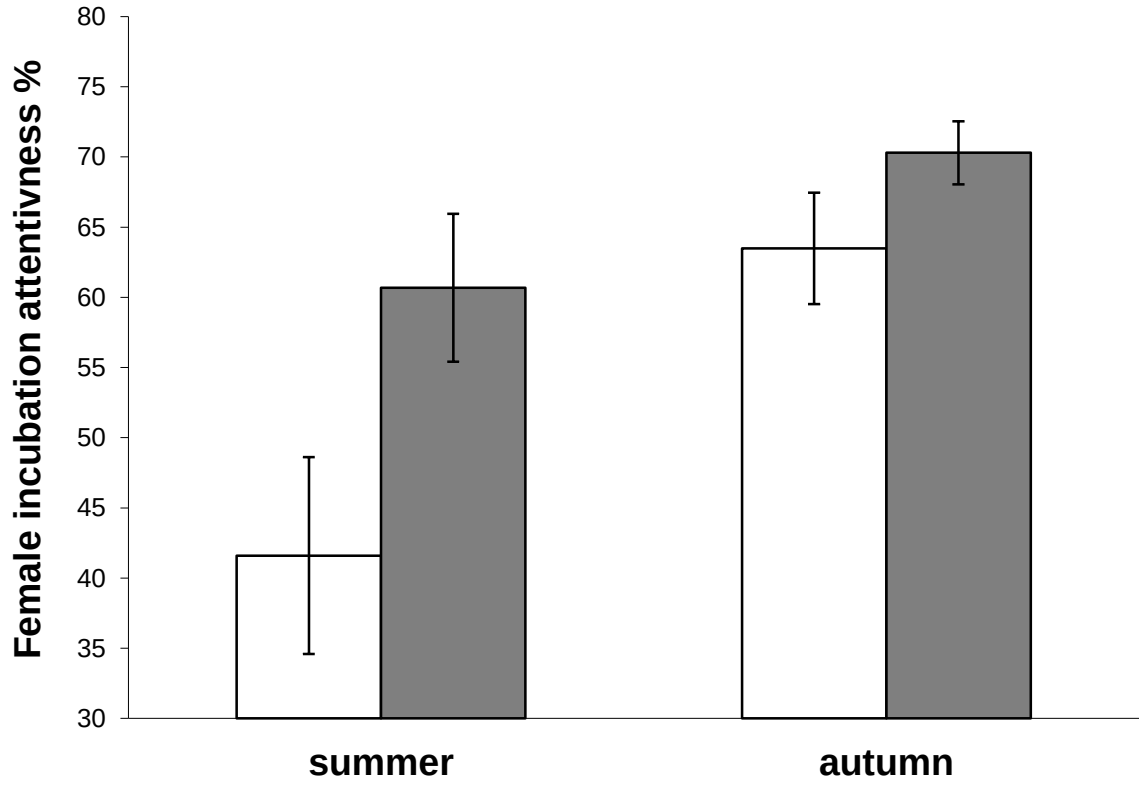


Figure 3.1: Mean (±SE) female incubation attentiveness in inbred (open bars) and control (filled bars) females in first and second replicates expressed as a percentage of total incubation time by pairs. There was no interaction between replicate and inbreeding status on female incubation attentiveness (non-significant inbreeding status * replicate: t=1.43, n=29, p=0.196 in the model shown in Table 3.2b).

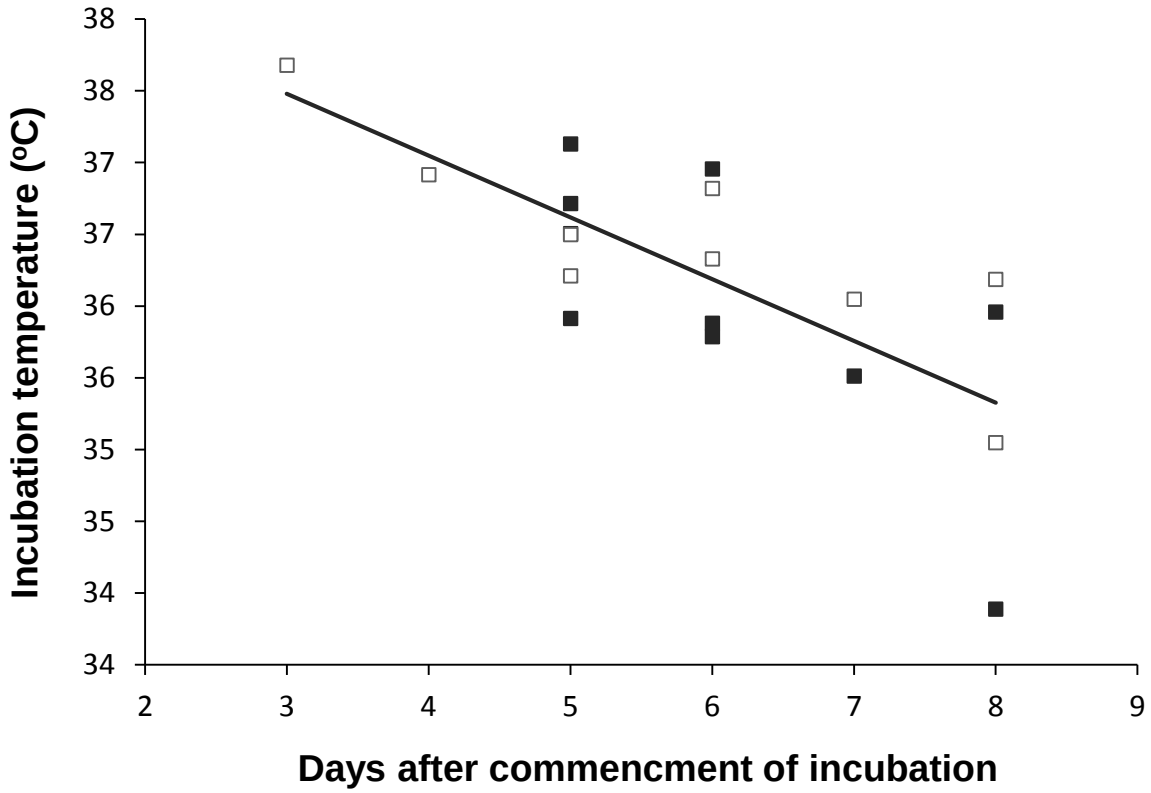


Figure 3.2: Mean steady-state incubation temperature of a sub-sample of 19 females (9 inbred females, open symbols and 10 control females, filled symbols) from 15 different families declined with increasing day of incubation (Table 3.2c).

3.5 Discussion

The aim of this study was to investigate whether maternal inbreeding caused a decline in incubation attentiveness or mean incubation temperature. I further investigated whether inbreeding in the foster mother lead to a decline in embryo viability. I found a significant inbreeding depression in the incubation attentiveness of captive female zebra finches that were derived from brother x sister pairings when compared to control birds, which is one of the first estimates of inbreeding depression for parental effort. It had been suggested that inbred female Seychelles warblers, *Acrocephalus sechellensis*, may invest less in parental care during early embryo development, either in egg formation or in incubation compared to control females (Richardson *et al.* 2004). My findings on zebra finches now confirm that the females' inbreeding status can indeed be associated with reduced levels of parental care, and, specifically, reduced levels of incubation expenditure. However, despite the reduced incubation attentiveness of inbred females, total incubation between nests of inbred and control females did not differ, implying that male partners were able to fully compensate for their female's reduced incubation attentiveness. The steady-state incubation temperature at which females incubated their eggs also did not differ between inbred and control females, suggesting that only the time available for incubation, but not ability to warm the eggs, was affected in inbred females. There was, however, no statistically significant effect of the female's inbreeding status on embryo growth and survival in a captive environment, although the biological effects observed in this study could be sufficient to result in fitness costs in the wild.

Although the precise level of inbreeding in my two groups of zebra finches is not known, background inbreeding levels of captive zebra finch populations has been estimated to be small and similar to many wild avian populations (Forstmeier *et al.* 2007). The true inbreeding coefficient of brother-sister pairs might be lower than f=0.25 if there is already some background inbreeding, but given the small levels of background inbreeding in captive zebra finch populations, the difference in inbreeding coefficient between treatments is likely to be close to f=0.25. The inbreeding depression in my study should therefore be comparable with high inbreeding in the wild. For incubation attentiveness, my study estimates a coefficient of inbreeding depression of 17% at full-sibling mating (although it may be substantially higher as the GLM estimated a decrease of 30.9% ±15.9 when statistically controlling for other factors). A comparative analysis of the results from 54 animal species revealed that life-history traits showed a median coefficient of inbreeding depression at f=0.25 of 11.8%, while the median inbreeding depression of

morphological traits was only 2.2% (DeRose & Roff 1999). Thus my findings for incubation attentiveness are in agreement with the higher level of inbreeding depression for life-history traits. Life-history traits, such as those that affect survival and reproduction, are particularly susceptible to inbreeding depression as they are likely to be under strong selection, have a high ratio of dominance to additive variation and represent a wide mutational target due to the large numbers of loci influencing such traits (Merilä & Sheldon 1999).

Inbred females may show lower reproductive expenditure for at least two, not mutually exclusive, reasons. Firstly, in female zebra finches inbreeding has been shown to be associated with reduced fat scores, a standard metric of calvicular and abdominal fat (Bolund *et al*. 2010), and poorer maternal condition is associated with lower incubation attentiveness (Gorman & Nager 2003).I did not measure the condition of females over the course of incubation, however, inbred birds in my population significantly skeletally smaller than control birds. Furthermore, inbred birds have significantly lower mean body mass when measuring a large sample of male and female zebra finches (unpublished data). Similar results have previously been reported in zebra finches (Bolund *et al*., 2010). During a demanding activity, such as reproduction, condition of inbred individuals may also deteriorate faster than control individuals (Jimenez *et al.* 1994).

Secondly, inbred individuals may have a higher resting metabolic rate than outbred individuals (Ketola & Kotiaho 2009a). If an organism has a limited ceiling of how much energy it can spend, and this is not affected by inbreeding (Ketola & Kotiaho 2009a), then inbred females may have less energy available for activities other than self-maintenance compared to control individuals. Indeed, I found that female zebra finches in this population had higher resting metabolic rates than control females (chapter 4.4.1) which could result in a reduction in energy available for incubation in inbred females. Both explanations could mean that inbred birds require longer periods between incubation bouts to replenish resources. These mechanisms could also cause the birds to more rapidly reach a critical threshold in energy stores, at which they have to stop incubating and resume foraging.

Despite the reduction in incubation attentiveness, inbred females did not incubate their eggs at a lower temperature than control females. While data could only be obtained from a small sub-sample of individuals due to logistical constraints, and therefore the statistical power is low, the difference in temperature is, if anything, slightly higher ($<0.4^{o}C$) in

inbred compared to control females. The slightly higher incubation temperature in inbred birds may have resulted from the higher metabolic rate of inbred females compared to control females (chapter 4.4.1). Incubation temperature may be constrained by the requirement of the embryo to optimally develop within a narrow set of temperatures (Webb 1987). The higher energy expenditure per unit time may have caused them to reduce the time they spend incubating, but, as a side effect, kept incubation temperature high.

If one parent reduces its parental expenditure, as the female reduces her incubation attentiveness in my study, then theoretical models of division of labour between parents predict that its partner should show partial compensation (reviewed in Houston *et al.* 2005). Indeed, a meta-analysis of empirical studies of parental care found that, on average, parents responded with only partial compensation if their partner reduced their share of parental care (Harrison *et al.* 2009). The total amount of time that eggs were incubated by either the male or female parent, however, did not differ between nests of inbred and control females. This suggests that the males in my study fully compensated for the reduction in their partner's incubation attentiveness. Previous studies also demonstrated full compensation (e.g. Mrowka 1982; Sanz *et al.* 2000; Osorno & Székely 2004) and this may have occurred if the risk of total breeding failure increases with a decline in parental care (Jones *et al.* 2002). For zebra finches a low total incubation attentiveness can negatively affect embryo viability (Gorman *et al.* 2005a). Alternatively, the full compensation observed here might have only been possible because of the relatively benign laboratory conditions, whereas under the more stressful conditions in the wild, where incubation attentiveness is generally lower than in captivity (Zann 1996), the male might not have been able to fully compensate for their partners reduced incubation attentiveness.

Zebra finch eggs incubated by inbred females had an 8.5% lower hatching success than eggs incubated by control mothers; however, this difference was not statistically significant. This may be because of the low statistical power of the test with only 29 nests and so a larger sample size may have revealed an effect on hatching success. Other studies reported a coefficient of inbreeding for hatching success at f=0.25 between 3 and 93% (Sittmann *et al.* 1966; Keller 1998; Marr *et al.* 2006). The two most extreme values, 3 and 93%, come from song sparrows, *Melospiza melodia*, in periods without rain and with heavy rain, respectively (Marr *et al.* 2006). The (non-significant) effect of maternally mediated inbreeding depression in hatching success found in my study is towards the lower

end of the distribution observed in studies of wild birds, and may be consistent with the idea that inbreeding depression is lower in more benign environmental conditions (Armbruster & Reed 2005).

Furthermore, the effects observed in field studies are presumably the result of the cumulative effects of dam inbreeding on both egg size (Sittmann *et al.* 1966; Sewalem *et al.* 1999; chapter 2) and incubation behaviour (current study). In my study I only observed the effects of maternal inbreeding on hatching success through differences in incubation attentiveness of inbred vs. control females. This might explain the relatively small effect of inbreeding on hatching success as the cumulative effects of maternal inbreeding on both egg production and incubation behaviour would presumably have a greater effect on hatching success than either of these mechanisms acting alone. The effects of maternal inbreeding on incubation effort may therefore be expected to increase under the harsher conditions of the wild, especially when combined with the effect of maternal inbreeding on egg production (chapter 2.4.1). Nonetheless, an inbreeding depression of 8.5%, even if statistically insignificant, is still high compared to many other traits (DeRose & Roff 1999) and may be biologically significant in combination with effects at other life-history stages.

My study also showed associations between incubation and some of the other variables I statistically controlled for. Firstly, I found differences in reproductive expenditure between the two replicates, wherein female zebra finches breeding in the first (summer) replicate showed lower incubation attentiveness but laid larger clutches than females breeding in the second (autumn) replicate. Although zebra finches are opportunistic breeders and are able to breed throughout the year (Zann 1996), they still show some seasonal breeding patterns (Perfito *et al.* 2007). In captivity, zebra finches have been shown to have higher reproductive investment, including larger clutch size in the spring and summer compared to the autumn and winter, even under apparently constant laboratory conditions (Williamson *et al.* 2008). This is consistent with the larger clutches I found in the first (summer) compared to the second (autumn) replicate. The lower egg production expenditure in the autumn may have resulted in the females maintaining better condition at the start of incubation, leading to their higher incubation attentiveness.

Secondly, females derived from second and third broods had lower incubation attentiveness, maybe due to poorer rearing conditions provided by parents that have already raised a previous brood (Burley *et al.* 1992) and their long-term consequences on subsequent reproductive expenditure (Gorman & Nager 2004; Naguib & Gil 2005;

Tschirren *et al.* 2009). Given that female incubation attentiveness declined from first to subsequent broods but first broods were more frequent in the first replicate, the two effects (brood and replicate) are most likely independent influences on incubation attentiveness but act in opposite directions.

Thirdly, there were also interactive effects of replicate, laying position and clutch size on hatching mass. The relationship between hatching mass and laying position depended on replicate: hatching mass increased with laying position in the first (summer) replicate but showed a non-significant decline with laying position in the second (autumn) replicate. In zebra finches, offspring size generally increases with laying position and late offspring come from larger eggs and hatch as heavier hatchlings, possibly to attenuate the effects of hatching asynchrony by making later hatched chicks better able to compete with their larger siblings (reviewed in Griffith & Buchanan 2010). A similar interaction between laying position and season was also reported by Williamson *et al.* (2008) where offspring size increased with laying position in spring, summer and autumn but not in winter. Hatching mass was positively associated with clutch size in the autumn but not in the summer and this may be a result of the smaller clutches in autumn, which may have meant females were not producing eggs to full capacity and were therefore more able to invest in larger egg size when laying relatively large clutches.

In conclusion, this study shows maternally-mediated inbreeding depression in the level of parental care in incubating zebra finches. While the underlying mechanism is unclear, the reduction in incubation expenditure of the inbred parent may explain, at least for species with uniparental care and/or sub-optimal rearing conditions, the widely observed negative effect of parental inbreeding on hatching success. Other aspects of parental expenditure, such as egg formation and offspring provisioning may also detrimentally influence offspring viability of inbred mothers, but these traits remain yet to be fully investigated. If similar inbreeding depressions apply to other stages of parental care (egg formation, offspring provisioning), the combined effects across all stages could contribute substantially to a reduction in fitness of inbred mothers (Frankham *et al.* 2002).

4 The Effect of Inbreeding on Resting Metabolic Rate

4.1 Abstract

Inbred animals have previously been shown to have reduced growth rate, body size and condition indices. They also reproduce less successfully with lower parental care and reduced survival of their offspring compared to offspring of, non-inbred, control individuals. One possible reason for this effect is that inbreeding may affect the organism's metabolic function, resulting in higher metabolic maintenance costs. I investigated whether inbreeding leads to an increase in metabolic costs of self-maintenance in female zebra finches in terms of resting metabolic rate. In order to test the effect of inbreeding on resting metabolic rate, I measured resting VO_2 rates of 19 inbred and 22 control female zebra finches, *Taeniopygia guttata*. I found a higher mass-corrected resting metabolic rate in inbred birds compared to control birds and an increase in resting metabolic rate with birds that were more difficult to catch. I also measured the size of organs (heart, liver and pectoral muscle) and found that inbred birds had larger central organ mass (heart plus live) than control birds for their body size but showed no change in peripheral organ mass (pectoral muscle). This is in line with previous studies that have suggested that central organ mass, but not peripheral organ mass, increases with resting metabolic rate (e.g. Daan *et al.* 1990). Resting metabolic rate correlated positively with central organ mass and negatively with peripheral organ mass. If maximal metabolic rate is not also increased by inbreeding, then increased resting metabolic rate could lead to a decrease in the availability of energy for energetically demanding activities such as growth or reproduction. This result could have important implications for life-history traits and, ultimately, fitness of inbred animals.

4.2 Introduction

Resting metabolic rate is of central interest to life-history theory and has been associated with a range of life-history traits. These include traits such as dominance (Hogstad 1987; Bryant & Newton 1994; Metcalfe *et al.* 1995; Yamamoto *et al.* 1998; Senar *et al.* 2000; Sloman *et al.* 2000), date of first feeding (Metcalfe *et al.* 1995), growth (Yamamoto *et al.* 1998) and maternal dominance (Burton *et al.* 2013), survival (Larivée *et al.* 2010; Artacho & Nespolo 2009; Jackson *et al.* 2001) and reproductive success (Nilsson and Råberg 2001; Blackmer *et al.* 2005). If the level of inbreeding in an animal affects resting metabolic rate it could therefore have important implications for the fitness of inbred animals. Inbreeding has been found to affect a wide variety of life-history traits such as survival and fecundity (Keller & Waller 2002). There is evidence that inbreeding depression has a greater impact on traits closely associated with fitness than on other traits (DeRose and Roff 1999). Increased inbreeding depression of fitness traits is thought to be due to the fact that such traits are under the control of many loci, each exerting a small effect, and therefore fitness-related traits represent a wide mutational target (Merilä and Sheldon 1999). Inbreeding depression is thought to be predominantly caused by the unmasking of rare deleterious alleles (Charlesworth & Willis 2009) and so a wider mutational target is predicted to lead to higher levels of inbreeding depression. The underlying mechanisms of inbreeding depression on survival and fecundity are unclear. Inbreeding may affect important systems within the body that have a wide mutational target such as immune function (Coltman *et al.* 1999; Hawley *et al.* 2005; Whiteman *et al.* 2006; Acevedo-Whitehouse *et al.* 2009) or ability to acquire and process resources (Jiménez *et al.* 1994; Potts *et al.* 1994), which may result in either reduced survival or fecundity.

Metabolic rate shows additive genetic variance (Rønning 2007), and is strongly related to fitness; as such it is expected to show a strong inbreeding depression like other fitness-related traits (DeRose & Roff 1999). Several studies have found a correlation between heterozygosity (often used as a proxy for inbreeding) and resting metabolic rate (RMR). Negative correlations between heterozygosity and resting metabolic rate have been found in molluscs (Garton 1984; Rodhouse & Gaffney 1984), salamanders, *Ambystoma tigrinum mavorium* (Mitton *et al.* 1986) and trout, *Salmo gairdneri* (Danzmann *et al.* 1987) whereas Carter *et al.* (1999) found a positive correlation between heterozygosity and resting metabolic rate in house mice, *Mus domesticus*. However, heterozygosity is often only loosely associated with inbreeding coefficient and so these correlations could reflect alternate processes other than inbreeding, such as associative overdominance (Pemberton

2004; Taylor *et al.* 2010), but this is disputed (Forstmeier *et al.* 2012; Hemmings *et al.* 2012). However, direct evidence of a relationship between inbreeding and metabolic rate based on pedigree information is rare. Richardson *et al.* (1994) found lower basal metabolic rate (BMR) and higher non-shivering thermogenesis in wild house mice and wild/laboratory hybrid crosses compared to laboratory mice. However, in this study it is difficult to distinguish the effects of inbreeding from other genetic processes such as adaptation to the laboratory environment. A study by Ketola & Kotiaho (2009a) on *Gryllodes sigillatus* crickets found an increase in resting metabolic rate with the level of inbreeding coefficient, *f*. However, the maximal metabolic rate was not influenced by inbreeding, which led to a decrease in their ability to allocate energy to activities other than self-maintenance, for example sexual signalling (Ketola & Kotiaho 2009b). Rantala & Roff's (2006) study on another cricket, *Gryllus firmus*, however, found no association between inbreeding and metabolic rate, but, unlike the study by Ketola & Kotiaho (2009a), this study compared inbreeding within historically inbred lines and so purging of deleterious alleles may have negated the effect of inbreeding on metabolic rate.

If inbreeding has an effect on the resting VO_2 of animals, it may also be expected to affect the size of metabolically active organs. Between avian species, metabolic rate tends to increase with the size of the most metabolically active organs such as the heart, liver and kidneys, presumably because larger organs require large amounts of energy for maintenance (Daan *et al.* 1990). However, the relationship between central organ mass and metabolic rate within species is not clear. Some studies have found a positive relationship between central organ mass and basal metabolic rate (Konarzewski and Diamond 1995; Meerlo *et al.* 1997; Chappell *et al.* 1999) whereas others have found no such relationship (e.g. Steyermark *et al.* 2005 (except kidney mass); Chappell *et al.* 2007; Norin and Malte 2012). However, the same association was not seen for peripheral organ mass between avian species (Daan *et al.* 1990), however, both positive (e.g. Chappell *et al.* 1999) and negative (e.g. Russell and Chappell 2007) associations have been found between BMR and peripheral organ mass with species.

Little research has been carried out investigating the effects of inbreeding on metabolic rate in vertebrates and, to the best of my knowledge, no studies have been carried out on birds. Here I compare resting metabolic rate, measured with open-flow respirometry, between offspring from brother-sister pairs (inbred females) and offspring raised at the same time from unrelated pairs (control females) of zebra finches *Taeniopygia guttata*. I predict that inbred females will have a higher resting metabolic rate than control females.

Elevation of resting metabolic rate may be associated with increased size of the metabolically active organs (e.g. Konarzewski & Diamond 1995; Meerlo *et al.* 1997; Chappell *et al.* 1999) and so inbreeding may also lead to an increase in the size of the heart and liver. As resting metabolic rate can have significant impacts upon many life-history traits, inbreeding depression in resting metabolic rate could have important implications for our understanding of the mechanisms underlying inbreeding depression. I investigated the following questions;

- Does inbreeding lead to an increase in resting metabolic rate of female zebra finches?

- Does the size of the central and peripheral organs correlate with resting metabolic rate in the zebra finch?

- Does inbreeding lead to an increase in the size of the metabolically active organs?

- Does resting metabolic rate reflect the ability of birds to evade capture?

- Does inbreeding affect the ability of birds to evade capture?

4.3 Materials and Methods

4.3.1 Production of Experimental Birds

The study was carried out on captive zebra finches held at the University of Glasgow. An overview of the experiments can be seen in fig. 1.1, section 1.11 and appendix one. In the winter 2008/09 I bred inbred and control zebra finch females from my stock of several hundred individuals with known pedigree since 2006; this stock is regularly replenished with birds from other populations (e.g. pet shops) in order to maintain genetic diversity. Inbreeding in captive zebra finches has been shown to be low and similar to that found in many wild avian populations (Forstmeier *et al.* 2007). To obtain inbred birds, I paired full-sibling brothers and sisters; control birds were created by pairing males and females from my stock population that did not share any grandparents (see chapter 2.3.1 for more details). Inbred and control females, selected for the current study, all had previous breeding experience in 2009 (chapters 2 and 3). Prior to the current study, female zebra finches were housed in a single sex group of 45 in a 2m x 1.8m x 1.8m aviary in a controlled temperature room (ambient temperature 21.39^{o}C±SD1.04) and kept on a 10:14 hour light:dark cycle (daylight-spectrum fluorescent tubes, Arcadia Bird Lamp FB36), which was gradually increased to 14:10 hours over several weeks previous to the measurement of metabolic rate to bring them into breeding condition. Birds were given *ad libitum* access to seed (Haith's, Lincolnshire, UK) and millet spray suspended from the ceiling. In addition, the birds were supplemented with dried hens' eggs and organic greens (e.g. spinach) once a week (see Hill *et al.* 2011 for more details).

4.3.2 Measurement of Metabolic Rate

Resting metabolic rate (RMR) was measured as O_2 consumption (VO_2) for all females that were used in the study on egg production and the study on incubation behaviour (chapters two and three, fig 1.1, section 1.11 and appendix one) and were still alive at the time of RMR measurement. This sample consisted of 19 control females (from 13 families) and 22 inbred females (from 13 families). Similar proportions of inbred and control females therefore survived to this point from my original experimental females (χ^2_1=0.271, p=0.602, χ^2 test). Respiratory measurements were made between the 18th of May and the 1st of June 2011 when the females were between 2.10 and 2.41 years old (inbred: 2.30±0.08 (SD) years, n=22; control; 2.27±0.11 (SD) years, n=19; t=1.20, n=41, p=0.237). I measured metabolic rate for four females (2 inbred and 2 control) at a time. The birds to be measured were removed from the holding aviary (using a small net or by hand) the day

before the measurement took place and placed in smaller cages (1.2mx0.4mx0.4m) within the same room until the next day. I ensured that inbred and control birds were measured evenly throughout the experimental period by measuring two inbred females and two control females per day. If I caught more than two birds of the same treatment group before having caught two of the other group, surplus birds were placed back in the flight aviary to be used on another day. Since there were more inbred females than control females, there were three days when two inbred females and only one control female were used and these days were randomly spread over the measurement period (runs 6, 8 and 11). The order in which the birds were caught was recorded in order to establish whether there was any relationship between the birds' ability to evade capture and resting VO_2. These results will not be influence by the fact that I took equal numbers of inbred and control females each day for the experiment, as if the wrong ratio of birds was caught, extraneous birds were placed back in the flight cage, but their original rank capture order was recorded. Therefore, this also allowed me to test whether inbred birds differed in rank capture order compared to control birds.

For the purpose of measuring VO_2, birds were in an open flow respirometry system (Lighton & Halsey 2011; Fig. 4.1) for a period of 2-2.5 hours, under a Home Office Licence at the University of Glasgow. For each run, five identical respirometry chambers of 12cm x 11cm x 14cm high were used. The four chambers, that contained one bird each, were equipped with a perch for the birds to use as this was considered less stressful than sitting on the floor (Graham Law, personal communication). Birds used this perch throughout the measurement period. Respirometry chambers were contained within an incubator (Labheat Incubator, Borolabs, Basingstoke) in order to maintain the temperature at 28^{o}C, which is within the zebra finches' thermal neutral zone (Calder & King 1963). The incubator was darkened with a blackout curtain to keep the birds calm whilst inside the chambers. A fifth chamber was left empty as a control for measuring baseline VO_2; this chamber had the same internal air volume as the experimental chambers. Outside air entered the system via a pump (Gast Manufacturing Inc., USA) and passed through a scrubber containing alternating Drierite (Drierite with indicator, 8 mesh; Sigma, 238988) to remove water, and Ascarite (Sodium Hydroxide Coated Silica, 20-30 mesh; Sigma, 223921) to remove carbon dioxide (Figure 4.1). Air passed through a manifold (Sable Systems MF-8 Airflow Manifold) that controlled airflow into the five respirometry chambers. A multiplexer (Sable Systems Respirometer Multiplexer, Version 2.0) received airflow from all chambers and alternated which stream to pass on to a mass flow unit and the oxygen analyser. The exact volume of air passing onto the oxygen analyser was

measured using a mass flow unit (Sierra Instruments Inc., The Mass Flow Company), controlled by a mass flow controller (Sable Systems 2-Channel Mass Flow Controller, Version 1.1). The air then flowed through another scrubber to remove water and carbon dioxide produced by the birds before entering the oxygen analyser (Sable Systems, FC-1B O_2 Analyser), which was connected to a data logger (Sable Systems International, Universal Interface, UI2) that stored and relayed data to a laptop computer. I calibrated the O_2 analyser using dried outside air.

On the morning the measurements of resting metabolic rate were taken, females were removed from their temporary holding cages at around 9am and were weighed to the nearest 0.01g before being randomly assigned to a respirometry chamber, using a random number generator. Measurements began at around 10am (range 9:40-10:15am). Once all chambers were sealed, the system equilibrated on the control chamber for 20 minutes to allow stabilisation and calibration of oxygen (20.8% O_2) using dry outside air; this also allowing time for the animals to habituate to the chambers. The outflowing air from the first respirometry chamber was then measured for 586s before switching to the next chamber. Once all four respirometry chambers containing birds were measured the cycle started again beginning with the control chamber. At the end of the second cycle a final switch to the control chamber was done, the flow rate was allowed to stabilise and two final baseline oxygen readings were taken, one after two minutes and one after 15 minutes; the mean of these two readings was taken as the final baseline oxygen reading. VO_2 was measured every second for two periods of 586s for each bird. The average oxygen concentration over each measurement period was obtained using Sable Systems software (Sable Systems Europe GmbH, Berlin, Germany) after the flow rate stabilised at around 495ml/min. Measurements of baseline O_2 were taken from the empty control chamber at the start, middle and end of recordings. To account for potential temporal changes in baseline O_2, a trend line, from the first baseline O_2 reading to the second (middle) O_2 reading was taken to calculate a time-dependent baseline O_2 for the first set of measurements of VO_2. A second trend line from the second (middle) O_2 reading to the final baseline O_2 reading was used to calculate a time-dependent baseline O_2 for the second set of VO_2 readings. The two readings for each bird were highly correlated ($r=0.910$, $n=41$, $p<0.001$) and thus I averaged the two measurements of VO_2 to obtain a mean value of resting VO_2 for each bird. At the end of the measurement period birds were removed, re-weighed to the nearest 0.01g and transferred back to the holding aviary.

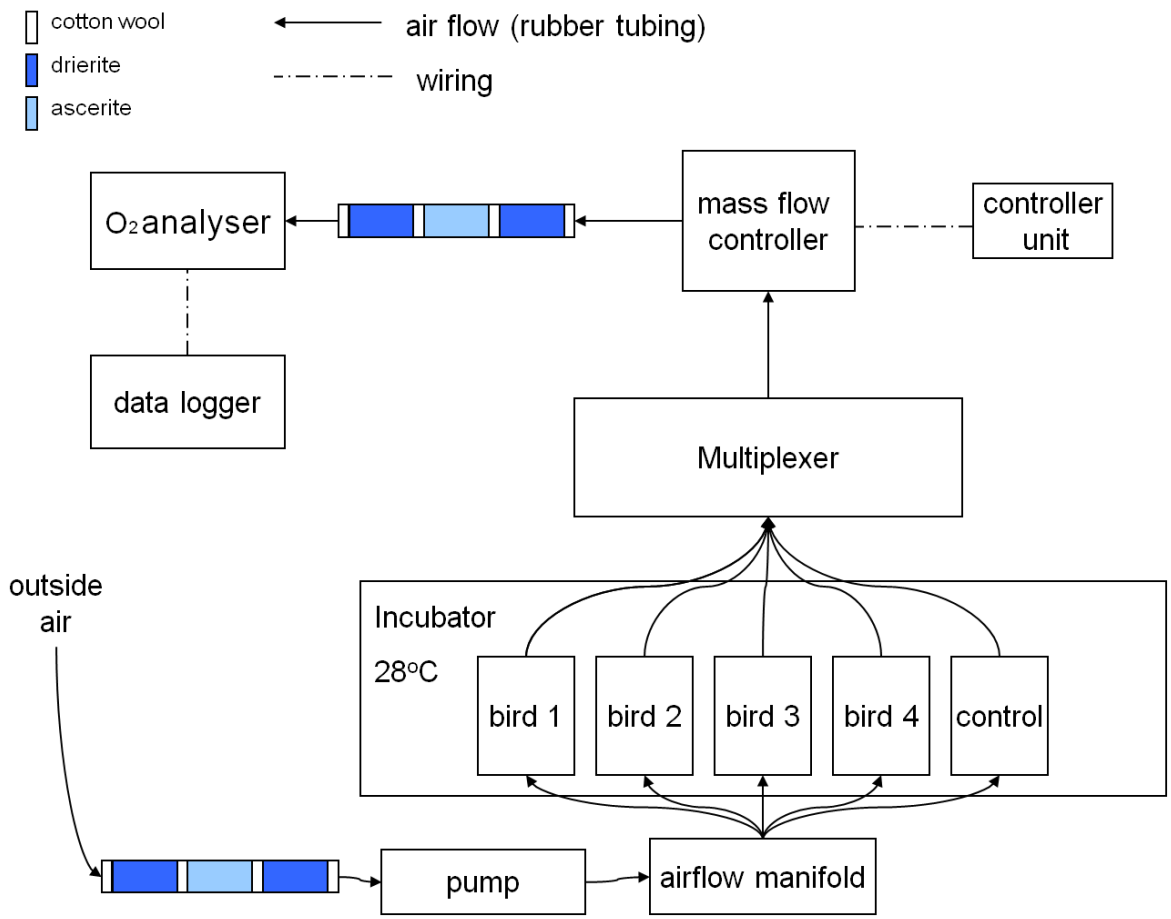


Figure 4.1 Diagram of the set-up of the open flow respirometry system. Air was pumped from outside of the building, via a tube leading to the roof of the building. Air was then scrubbed of water and CO_2 before entering the airflow manifold that led to the four experimental chambers and the empty control chamber. Air from the chambers was then fed into a multiplexer that switched the airflow between the five chambers sequentially. Airflow to the O_2 analyser was controlled by the mass flow controller and scrubbed of O_2 and CO_2 before finally entering the O_2 analyser. The readings were recorded by a data logger that fed the information to a computer where the readings could be read (see methods for details).

4.3.3 Body Composition

Approximately nine months after the readings of metabolic rate were taken all surviving females were weighed and then sacrificed by cervical dislocation. Following death, carcasses were immediately dissected. The pectoral muscles, heart and liver were removed, weighed to the nearest 0.01g, giving their wet weight, wrapped in foil and stored at -20ºC.

The frozen tissues were later defrosted and re-weighed to the nearest 0.001g. Defrosted samples were placed in a drying oven at 55ºC. Tissues were dried for a period of 24 hours by which time samples had reached a steady weight (this was established by weighing a sub-sample of three samples per tissue type every 4 to 8 hours). After drying, samples were removed from the oven and allowed to cool in a desiccator before being re-weighed to the nearest 0.001g, giving the dry weight. Following this, lipids were extracted from the dried tissue samples using petroleum ether extraction in a Soxhlet apparatus (Dobush *et al.* 1985). Lean (lipid-extracted) samples were then re-dried to constant weight once more before being allowed to cool and then weighed to the nearest 0.001g to determine lean dry mass of tissues.

4.3.4 Statistical Analysis

Since resting VO_2 scales allometrically with body mass (Feldman & McMahon 1983) it is necessary to correct for body mass in measurements of VO_2. The effect of body mass on VO_2 was statistically controlled for using a reduced major axis (RMA) regression rather than an ordinary least squares (OLS) regression. The OLS regression minimizes residuals along the y axis whilst assuming the x axis is free of measurement error. This assumption is often violated and so the OLS regression can lead to biased estimates whereas the RMA regression simultaneously minimizes residuals along the x and y axis and so avoids this problem (Forstmeier 2011). Zebra finches lost a significant amount of body mass during the recording of metabolic rate and thus there is some uncertainty of the exact body mass for each measure of VO_2. The RMA regression approach is therefore more suitable for correcting for body mass in this circumstance and has previously been used for modelling the effect of body mass on RMR in this species (Rønning *et al.* 2005). I calculated residual VO_2 as the residual values of observed VO_2 from the value predicted by the RMA given the average of the initial and final body mass during the RMR measurement period. Difference in residual VO_2 between inbred and control birds was tested using a general linear mixed model (*glmm*) with inbreeding status as a fixed factor, rank capture order as

covariate and family of origin as a random factor as some of the females were related (the 41 females originated from 26 different families).

Resting metabolic rate may be expected to increase with increasing size of metabolically active central organs (heart, liver and kidneys) (Daan *et al.* 1990). To represent the metabolically active organs heart and liver were added together to give a measure of central organ mass; kidneys of zebra finches were too small to measure accurately. Pectoral muscle mass was used to represent the main peripheral organ mass. Measurements of wet and lean central and peripheral mass were corrected for body mass using residuals of a RMA regression of mass immediately before death vs. central/peripheral organ mass immediately after death. I used these residual wet and lean masses (i.e. residuals from the RMA regression of body mass vs. wet and lean organ mass) when comparing organ size between inbred and control females in a general linear mixed model with inbreeding status as a fixed factor and family as a random factor. To explore the relationship between organ size and VO_2 I used a general linear mixed models with residual VO_2 as a response variable and wet or lean mass of central or peripheral organs as covariates and family as a random factor in each of the four models (residual VO_2 vs. residual wet central organ mass, residual VO_2 vs. residual lean central organ mass, residual VO_2 vs. residual wet peripheral organ mass and residual VO_2 vs. residual lean peripheral organ mass). Some of the females died of natural causes between the RMR measurements and when body composition was determined and therefore measurements of organ size were not available for all females with measurements on VO_2. Organ size data were available for 13 inbred (9 families) and 14 control females (10 families).

In all analyses, I started from a full model including the main effects and all possible two way interactions between the main effects. Non-significant terms ($p>0.05$) were removed sequentially, starting with interaction terms, until a minimum adequate model, containing only significant terms, was obtained. Only main effects and significant interaction terms will be shown. All data are presented as means ± SE unless otherwise stated.

4.4 Results

4.4.1 Resting VO_2

The differences in VO_2, body size and body composition between inbred and control birds are summarised in table 4.1, in terms of coefficient of inbreeding (i.e. percentage change in trait value with inbreeding of f=0.25), and effect size (Cohen's d) which is the difference in trait value, with inbreeding of f=0.25, divided by the pooled standard deviation. Although inbred females were 8.1% lighter than control females (Table 4.1) this difference was not statistically significant (*glmm* with family as a random factor: t=1.58, n=41, p=0.127). VO_2 increased with increasing body mass (OLS regression, t=2.30, n=41, p=0.037) and in order to control statistically for body mass, residual VO_2 was calculated as residuals from an RMA regression of VO_2 on body mass. Positive residual values from this RMA relationship indicate that the animal has a high resting metabolic rate for its body mass while negative residuals (above and below the solid line, respectively; Figure 4.2) indicate that the animal has a low resting metabolic rate for its body mass. Residual VO_2 was positively correlated with rank caught and inbreeding status (*glmm*, n=41: rank caught: t=3.77, p=0.002; inbreeding status: t=2.57, p=0.017; measurement date: t=1.39, p=0.186).

Table 4.1 Mean (±SE) traits of inbred and control zebra finch females. Coefficient of inbreeding depression (coefficient of ID) is the percentage change in trait value at an inbreeding coefficient *f*=0.25 and effect size of inbreeding was calculated as the mean difference in trait value divided by the pooled standard deviation of the two groups (Cohen's d). Inbreeding depression is calculated only for residual values of VO_2 and organ mass (i.e. residuals from an RMA regression of body mass vs. VO_2/organ mass). Traits showing statistically significant effects of inbreeding are highlighted in bold.

	Absolute		Residual		Inbreeding depression	
Trait	**Inbred (mean ± SE)**	**Control (mean ± SE)**	**Inbred (mean ± SE)**	**Control (mean ± SE)**	**δ (%)**	**Effect Size (Cohen's D)**
Body mass (g)	14.83 (0.32)	16.14 (0.49)	-	-	-8.12	-0.72
VO_2 (ml/min)	**0.91 (0.04)**	**0.86 (0.03)**	**0.07 (0.03)**	**-0.05 (0.04)**	**13.39**	**0.72**
Wet peripheral organ mass	3.12 (0.10)	3.278 (0.13)	-0.006 (0.122)	0.004 (0.09)	-0.31	-0.03
Lean peripheral organ mass	0.81 (0.03)	0.84 (0.04)	-0.017 (0.03)	0.0115 (0.03)	-3.45	-0.25
Wet central organ mass	**0.79 (0.05)**	**0.74 (0.03)**	**0.06 (0.02)**	**-0.056 (0.04)**	**15.17**	**0.99**
Lean central organ mass	**0.18 (0.04)**	**0.16 (0.04)**	**0.018 (0.01)**	**-0.017 (0.01)**	**21.16**	**0.99**
Wet heart mass	0.28 (0.05)	0.26 (0.05)	0.021 (0.01)	-0.019 (0.02)	14.58	0.77
Lean heart mass	0.05 (0.003)	0.04 (0.003)	0.005 (0.003)	-0.005 (0.003)	20.67	0.69
Lean liver mass	0.51 (0.03)	0.48 (0.03)	0.04 (0.02)	-0.05 (0.03)	18.23	0.88
Lean liver mass	0.13 (0.01)	0.11 (0.01)	0.007 (0.01)	-0.0077 (0.01)	12.33	0.46

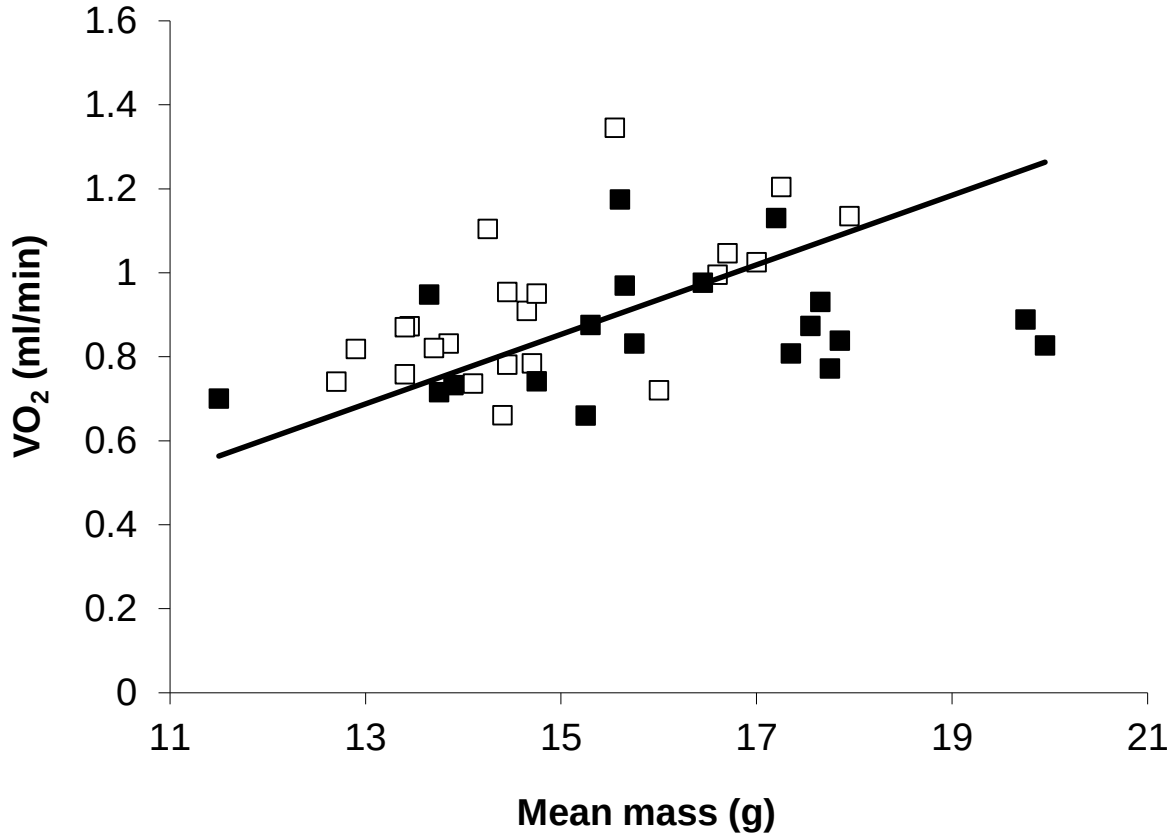


Figure 4.2 Relationship between body mass and VO2 for inbred (open squares; n=22) and control (filled squares; n=19) females. The solid line represents the slope of the reduced major axis regression (see methods).

4.4.2 Body Composition

Body composition also differed between inbred and control females at three years age, six months after VO_2 was measured (Table 4.1; Fig 4.3). Heavier females had larger organs and thus residual organ mass was calculated as the residuals of an RMA regression of organ mass on body mass. Residual central organ mass was significantly higher in inbred birds compared to control birds for both wet (*glmm* with family as a random factor; t=2.32, n=27, p=0.020; Figure 4.3a) and lean (*glmm* with family as a random factor; t=2.59, n=27 p=0.019; Figure 4.3b) tissues. Both residual heart and liver mass on their own showed the same difference between inbred and control females, although they were not statistically significant (Table 4.1; *glmm*s; wet heart mass: t=1.80, n=27, p=0.087; lean heart mass: t=1.34, n=27, p=0.196; wet liver mass: t=2.04, n=27, p=0.056; lean liver mass: t=1.74, n=27, 0.098). However residual peripheral organ mass was similar between inbred and control females for both wet (*glmm* with family as a random factor; stats; Figure 4.3c) and lean peripheral organ mass (*glmm* with family as a random factor; stats; Figure 4.3d).

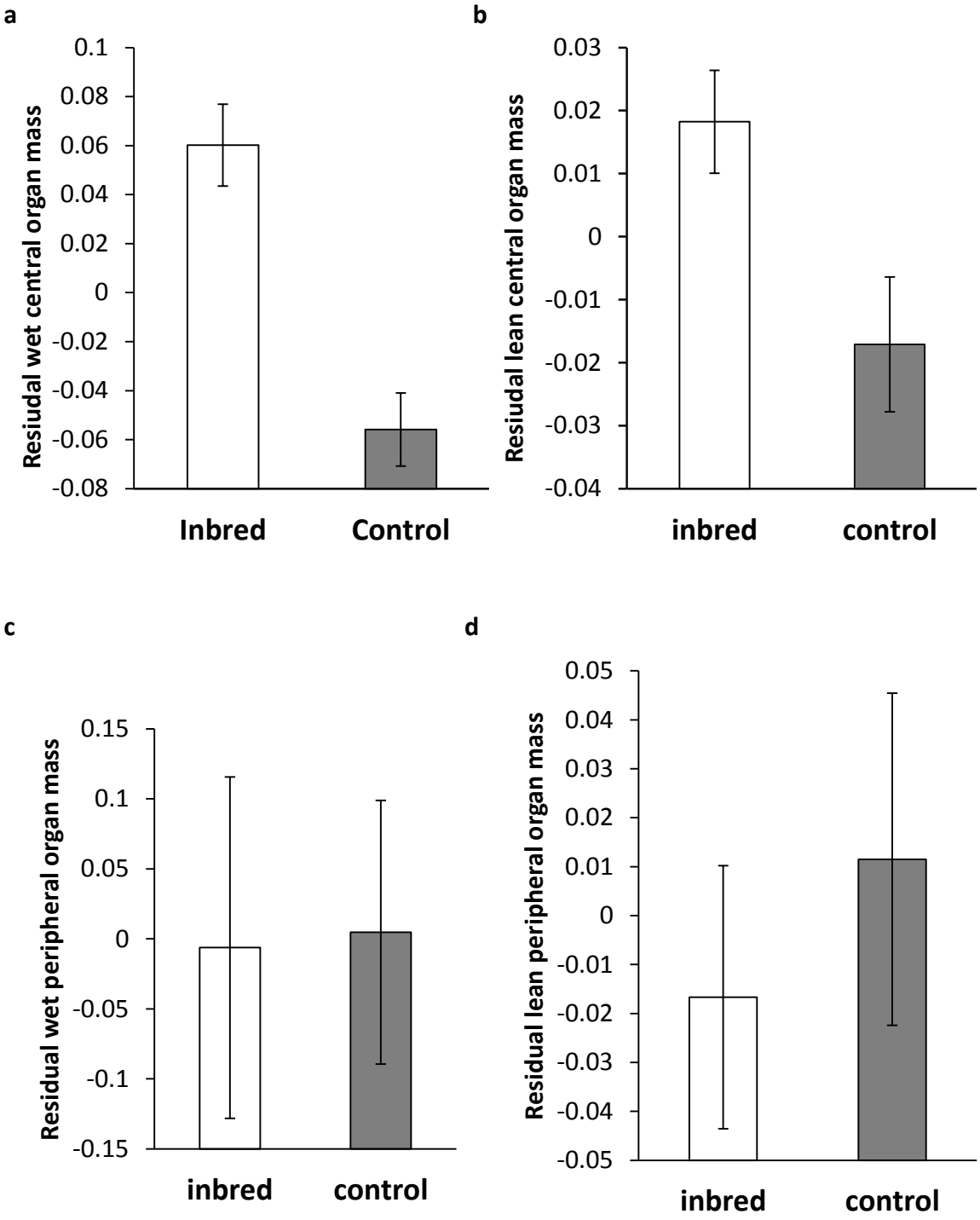


Figure 4.3 Residual central **(a and b)** and peripheral **(c and d)** organ mass for wet **(a and c)** and lean **(b and d)** tissue in inbred (n=13; open bars) and control (n=14; filled bars) females. **(a)** Wet residual central organ mass was significantly higher in inbred compared to control females. **(b)** Lean residual central organ mass was significantly higher in inbred compared to control females. **(c)** There was no effect of inbreeding on wet residual peripheral organ mass. **(d)** There was no effect of inbreeding on lean residual peripheral organ mass.

4.4.3 Resting VO_2 vs. Body Composition

Relative VO_2 was higher in birds with larger residual central organ mass (heart plus liver mass combined) for both wet mass ($t=2.55$, $p=0.038$; Figure 4.4a) and lean mass ($t=3.04$, $p=0.019$; Figure 4.4b). In contrast, residual VO_2 declined with increasing residual lean peripheral organ mass ($t=2.46$, $p=0.043$; Figure 4.4d) and wet peripheral organ mass ($t=1.961$, $p=0.091$ Figure 4.4c), although the later was not statistically significant.

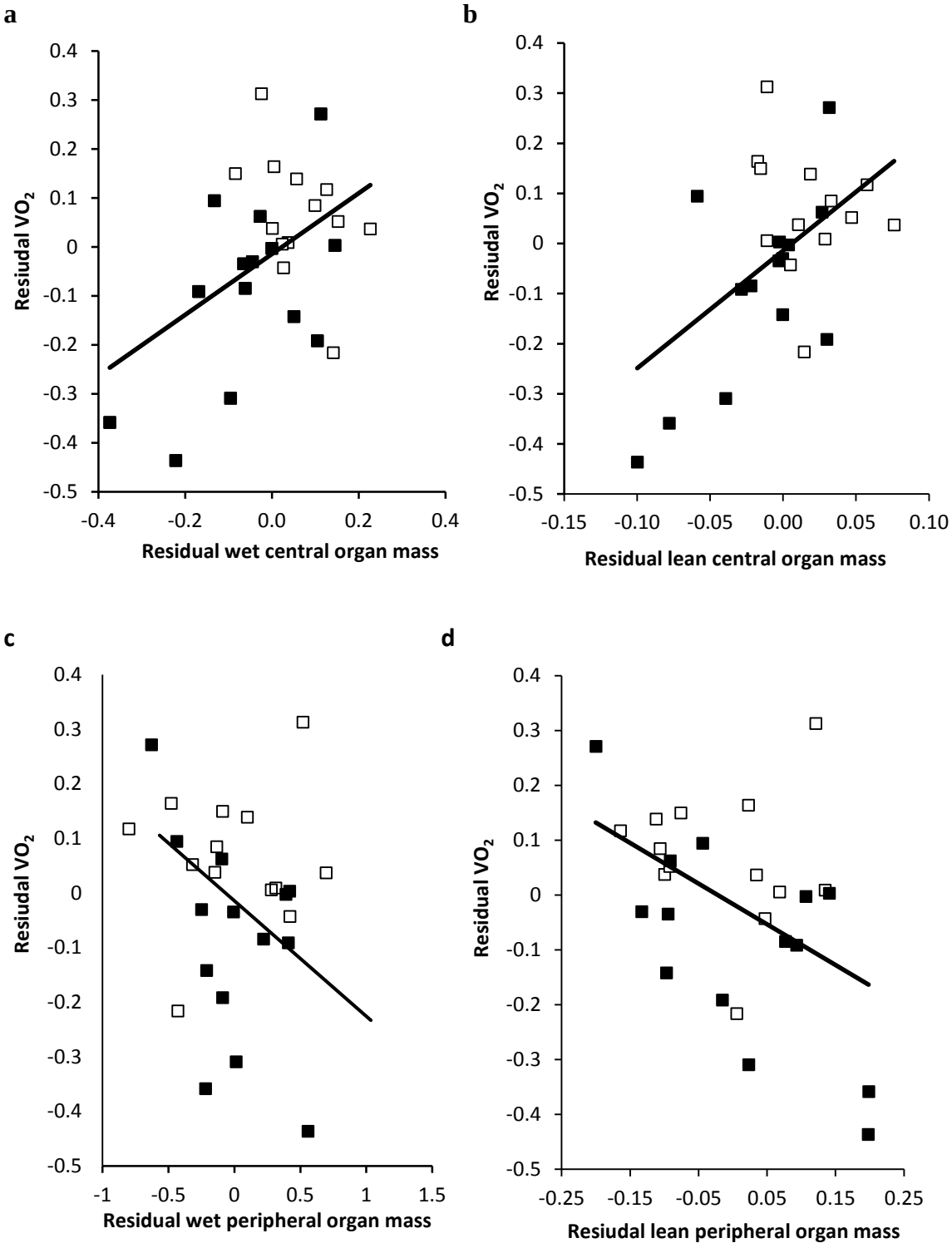


Figure 4.4 General linear mixed model of residual VO_2 consumption vs. (a) residual wet central organ mass, (b) residual lean central organ mass, (c) residual wet peripheral organ mass and (d) residual lean peripheral organ mass, n=27. In inbred (n=13; open squares) and control (n=14; filled squares). Residual are calculated from RMA regressions of body mass vs. VO_2/organ mass.

4.4.4 Resting VO_2 vs. Rank Capture Order

There was a significant positive correlation between rank capture order and residual VO_2 (*glmm* with family as a random factor; t=3.77, n=41, p=0.002; Figure 4.5). This indicated that birds that were harder to catch had generally higher resting metabolic rates than birds that birds that were easily caught. This effect is unlikely to be caused by differences in date as there was no change in absolute VO_2 readings over time (*glmm* with family as a random factor t=1.39, p=0.186). However, there was no difference in the rank at which inbred and control female were caught (Mann Whitney test, W=506.5, n=41, p=0.250).

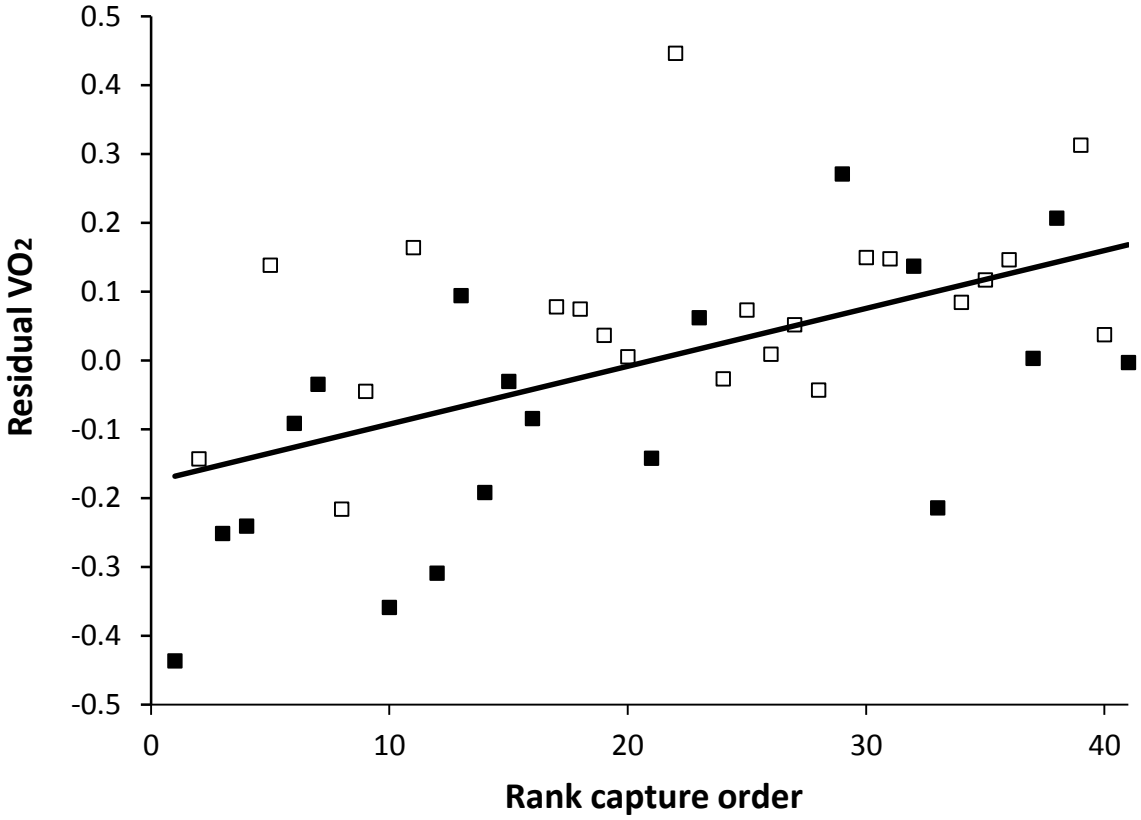


Figure 4.5 Regression of mass corrected VO_2 against the order in which the birds were caught (1-41). Low number indicate the birds that were easiest to catch, high numbers indicate birds that were difficult to catch. There was no effect of inbreeding on the order in which birds were caught.

4.5 Discussion

I investigated whether inbreeding lead to an increase in resting metabolic rate (RMR) of zebra finches (measured as VO_2 consumption of resting birds). I further investigated the relationship between RMR and the size of the central and peripheral organs and the effect of inbreeding on central and peripheral organ mass. Finally I explored the relationship between RMR, inbreeding and the ability of birds to evade capture. Resting VO_2 was significantly higher in inbred compared to control female zebra finches accounting for body mass. Although there was no statistically significant difference in body mass between inbred and control females, inbred females had a significantly larger central organ mass, when controlled for body mass, than control females. I also found that VO_2 was positively associated with the sum of the masses of the two central organs measured, liver and heart. This suggests that the increased maintenance oxygen consumption of inbred female zebra finches may be the result of larger central, metabolically active tissues. In contrast, the mass of peripheral tissues (i.e. pectoral muscle) was negatively associated with oxygen consumption but did not differ between inbred and control birds.

I estimated mean VO_2 for resting control birds of 3.3±SE0.12 ml O_2 / g h in my population. Studies by Rønning *et al.* (2005; 2007) found basal VO_2 between 39.8-41.4 ml O_2 / h per female zebra finches, which is slightly lower than our estimate, however, basal metabolic rate is expected to be lower than resting metabolic rate as the animal is fasted and females in Rønning *et al*'s study were 5-7% smaller than the females in our study. Studies by Vleck (1981) and Criscuolo *et al.* (2008) found similar mass-specific VO_2 to my study with VO_2 between 2.7 and 3.3 ml O_2 / g h for zebra finches under similar conditions to those in my study. My estimates of VO_2 in control birds are therefore very similar to those found in other studies of zebra finches.

Mass-corrected VO_2 of inbred birds was 13.4% higher than of control birds. Other studies had also suggested a link between metabolic rate and inbreeding. A few studies found correlations between heterozygosity and metabolic rate (Garton *et al.* 1984; Rodhouse & Gaffney 1984; Mitton *et al.* 1986; Danzmann *et al.* 1987; Carter *et al.* 1999). However, very few previous studies have directly compared metabolic rate between inbred and control individuals. Ketola and Kotiaho (2009a) investigated the effect of inbreeding on resting and maximal metabolic rate in male decorated crickets, *Gryllodes sigillatus*. Inbred crickets were found to have a higher resting metabolic rate than control crickets (Ketola and Kotiaho 2009a) but this was not found in a study by Rantala and Roff (2006) of

Gryllus firmus crickets. Richardson *et al.* (1994) suggested an increase in resting metabolic rate with inbreeding on the basis of a comparison between laboratory-bred and wild-caught mice but the observed differences in metabolic rate may be an adaption to laboratory conditions rather than being caused by inbreeding *per se*. This is, to my knowledge the first study to demonstrate an effect of inbreeding on resting metabolic rate in birds and one of the few studies in vertebrates.

The higher VO_2 in inbred individuals could be caused by a decline in the metabolic efficiency of inbred females compared to control females. For example, some energy is lost during metabolism due to mitochondrial proton leak, meaning that not all oxygen is coupled to ATP synthesis, and it is estimated that this can account for a substantial proportion of resting metabolic rate in animals (Rolfe & Brand 1997; White & Kearney 2013). Mitochondrial function has previously been found to affect basal metabolic rate (Tieleman et al. 2009). Hence, an increase in proton leakage could cause an increase in resting metabolic rate. It would be interesting to compare mitochondrial functions in inbred and control individuals. If there is a reduction in mitochondrial function in inbred individuals this may result in an increase in the size of the metabolically active organs (i.e. central organs) in order to cope with the increased energy requirements.

An alternative, but maybe not mutually exclusive, explanation for the higher metabolic rate could be the larger size of the metabolically active tissue in inbred female zebra finches. Although there was no statistically significant difference in body mass between inbred and control females, this probably reflects low statistical power, as in a larger sample of male and female zebra finches, inbred birds were significantly lighter than control birds (chapter 2.4.1). However, there were significant differences in the sum of heart and liver mass between inbred and control birds when correcting for body mass, but no differences in mean muscle mass between inbred and control females when correcting for body mass. The fact that central organ mass differed to a similar extent (15.2% for wet mass and 21.2% for lean mass) as VO_2 differed between inbred and control females (13.4%), suggest that this increase in VO_2 is not simply a result, of, for example, higher stress or activity levels in inbred females, and differences in body composition between inbred and control birds could play an important role the elevation of resting VO_2. I found a significant positive correlation between the mass of the central organs (heart plus liver) and residual RMR. A positive correlation between metabolic rate and mass of central organs has previously been described in mammals and birds (Speakman 2000; Daan *et al.* 1990; Konarzewski and Diamond 1995; Meerlo *et al.* 1997; Chappell *et al.* 1999) It is not clear

whether the increase in VO_2 found in inbred birds is caused by increased central organ size in these birds or, alternatively, that increased central organ mass is the result of increased VO_2 demands by inbred birds. Large metabolically active organs (such as the heart, liver and kidneys) require large amounts of energy to maintain them (Daan *et al.* 1990) and so an increase in central organ mass may necessitate an increase in VO_2. On the other hand, large central organs allow larger energy budgets (Konarzewski & Diamond, 1995) which may be associated with increased resting metabolic rates (Daan *et al.* 1990).

A further explanation for the difference in VO_2 between inbred and control birds could be developmental stress. Inbreeding of a similar magnitude to that in my study (around f=0.25) has been shown to act in a very similar way to poor early environmental conditions (Clarke *et al.* 1986; Roldan *et al.* 1998; Gomendio *et al.* 2000; Mazzi *et al.* 2002; Kristensen *et al.* 2005). Several studies have suggested that poor early developmental conditions can have long-term effects on metabolic characteristics. Increased brood size was associated with increased metabolic rate in zebra finches which was interpreted as a long-term effect of early nutritional stress (Verhulst *et al.* 2006). Criscuolo *et al.* (2008) went on to show that increased resting metabolic rate of individuals experiencing an early nutritional stress resulted from compensatory growth following a period of early nutritional stress rather than early nutritional stress *per se*. Blue tits *Cyanistes caeruleus* that experienced low incubation temperatures during their early development had higher resting metabolic rates (Nord and Nilsson 2011). The cumulative effects of deleterious mutations expected under inbreeding could act as developmental stressor in a similar manner to poor early developmental conditions and therefore cause changes in resting metabolic rate similar to those found for offspring experiencing sub-optimal conditions during early development.

In contrast the central organ mass, residual mass of peripheral tissues, i.e. pectoral muscle showed the opposite relationship, declining significantly with residual RMR. Similarly a study by Russell and Chappell (2007) found that muscle mass decreased with basal metabolic rate and combined central organ mass increased. The findings may suggest that in wild animals where food is not available *ad libitum* individuals with high residual RMR may be in poorer condition as they may have to spend a larger proportion of their energy intake into self-maintenance compared to individuals with lower RMR. High resting metabolic rate may result in less energy allocation to the growth and maintenance of pectoral muscle stores. High self-maintenance costs may even result in mobilisation of protein stores in muscle under high workloads. Although the birds were fed *ad libitum* in

the period before the measurements of resting VO_2 were carried out, they were housed in large flight cages and therefore had to expend energy to obtain food. Despite the decline in residual peripheral organ mass, there was no effect of inbreeding on the residual mass of the peripheral organs.

The increase in VO_2 in inbred females could have important repercussion for parental care. Inbred zebra finches reduced their reproductive expenditure in egg production and incubation attentiveness (chapters 2 and 3) compared to control birds. If maximal metabolic rate is not affected by inbreeding, and the few studies that looked at it suggest it is not (Richardson *et al.* 2004; Ketola and Kotiaho 2009a), then the difference between maintenance costs and the maximal rate of energy expenditure (the metabolic scope), which is available for activities other than maintenance, could be decreased in inbred individuals compared to control individuals. For example, a follow up study by Ketola and Kotiaho (2009b) found that sexual signalling in inbred decorated crickets, that had previously been shown to have to have increased metabolic rates but no change in total energy budgets (Ketola and Kotiaho 2009a), was reduced compared to control crickets.

Metabolic scope is an important metabolic characteristic with relevance to life-history traits (Speakman, 2000). Metabolic scope can be negatively correlated with resting metabolic rate for example in fish (Cutts *et al.* 2002) whereas, comparing across breeding birds of different species, Daan *et al.* (1990) found that basal metabolic rate increased with daily energy expenditure. Depending on whether metabolic scope increases or declines with resting metabolic rate, two theoretical predictions are possible (Nilsson 2002). According to the "increased intake hypothesis" individuals with a high capacity for energy intake may be able to support larger central organs. Conversely, the "compensation hypothesis" predicts that energy will be allocated amongst different activities (including self-maintenance) from a fixed amount of energy available to the organism and thus higher RMR will result in a reduced allocation of energy to other activities. Under the increased intake hypothesis RMR should vary positively with daily energy expenditure while the compensation hypothesis predicts a negative relationship between RMR and daily energy expenditure (Nilsson 2002).

Support for the increased intake hypothesis was produced in a meta-analysis of 22 avian species by Daan *et al.* (1990), which showed that basal metabolic rate significantly correlated with daily energy expenditure of breeding birds. They also found that basal metabolic rate and daily energy expenditure were both correlated with heart and kidney

mass. However, other studies leant support for the compensation hypothesis. Male Leach's storm petrels, *Oceanodroma leucorhoa* with lower basal metabolic rate had higher reproductive expenditures than males with high basal metabolic rates (Blackmer *et al.* 2005). Zebra finches, exposed to heavy workloads reduced their resting metabolic rates (Deerenberg *et al.* 1998). Increased metabolic rate could therefore either cause an increase or a decrease in energy availability for inbred female zebra finches. If increased resting metabolic rate were to result in a decrease in energy expenditure on reproduction; this could help to explain the findings of chapters two and three, i.e. that maternal investment in inbred females was reduced compared to control females.

Residual VO_2 showed a positive correlation with rank capture order (however rank caught did not differ between inbred and control birds). This implies that females with a high metabolic rate for their size were more difficult to catch than those with lower resting metabolic rate. Rank capture order has previously been shown to be repeatable in zebra finches (Birkhead *et al.* 1998). Presumably, this trait is indicative of how active the bird is, birds that were harder to catch spent more time flying, flew faster and/or were more manoeuvrable than those that were caught quickly. The increase in VO_2 with rank capture order may therefore reflect that more active birds had higher resting VO_2. However there was no effect of inbreeding on rank capture order, suggesting that whatever benefits are conferred by having a higher resting VO_2, in terms of their ability to evade capture, are not affecting inbred birds in the same way as control birds. This may imply that, for a given level of activity, inbred birds have to expend a higher level of energy on resting VO_2 than control birds.

In conclusion, I have shown differences in body composition and VO_2 between inbred and control zebra finches. The current findings could have important implications for our understanding of the underpinnings of maternally-mediated inbreeding depression as well as wider implications for the relationship between metabolic and life-history traits. The observed elevated maintenance costs of inbred birds could explain why inbred mothers allocate less energy to reproductive activities and thus their reduced parental care compared to control birds. As resting metabolic rate has been found to influence a wide range of life-history traits, these results could have implications for, survival, dominance and parental investment of inbred animals. Future studies should focus on the effects of inbreeding on other aspects of metabolism such as maximal VO_2 and daily energy expenditure. Future work could also focus on the relationship between inbreeding, RMR, energy allocation and parental effort to further illuminate how inbreeding can affect energy

budgets and also how inbreeding depression of metabolic traits might affect life-history traits and overall fitness.

5 General Discussion

5.1 Summary

As outlined in the general introduction, inbreeding can affect fitness in a variety of traits. Inbreeding can not only reduced fitness through reduced early survival of inbred individuals, but can also affect fitness later in life through inbreeding depression on the dam and sire (Keller & Waller 2002). Inbreeding has been found to reduce the early survival (particularly during embryogenesis) of offspring of inbred mothers, independent of the offsprings' own level of inbreeding (e.g. Sittmann *et al*. 1966; van Noordwijk & Scharloo 1981; Pulkkinen *et al*. 1998; Su *et al.* 1996; Margulis & Altmann 1997; Keller 1998; Moura *et al.* 2000; Marr *et al.* 2006; Farkas *et al.* 2007). However, the underlying causes of increase mortality of offspring of inbred females remains an open question. I examined the underlying causes of maternal inbreeding depression using female zebra finches, *Taeniopygia guttata*, that resulted from full-sibling pairings (inbred females) or unrelated pairings (control females) which were then paired to non-inbred, unrelated males.

Reduced offspring survival could be the result of reduced maternal investment. In chapters two and three I examined the effects of maternal inbreeding on two key stages of reproductive investment; egg production and incubation attentiveness. In chapter two I found a reduction in both egg mass and yolk mass with inbreeding in the laying female. In chapter three I found a reduction in incubation attentiveness with inbreeding in the incubating mother. These findings are novel and may shed light on the observations that maternal inbreeding can reduce early and long-term survival of the offspring of inbred individuals in wild populations (van Noordwijk & Scharloo 1981; Keller 1998; Jamieson *et al.* 2003; Richardson *et al.* 2004; Marr *et al.* 2006; Brouwer *et al.* 2007). Both egg mass and quality as well as incubation expenditure can affect survival and long-term phenotypic characteristics of offspring (reviewed in Krist 2011 and Webb 1987).

This lead to another open question; namely, what is the underlying cause of reduced maternal expenditure of inbred females? Reduced investment in reproduction could potentially be caused by higher maintenance costs resulting from elevated resting metabolic rate (e.g. Blackmer *et al.* 2005). This in turn, may be associate with increased size of metabolically active organs. In line with these predictions, in chapter four, I found

that resting metabolic rate (inferred from resting VO_2) and size of the heart and liver (both corrected for differences in body mass) of three year old females was significantly higher in inbred birds compared to control birds.

I also studied the effects of maternal inbreeding on both egg production and incubation on offspring viability and growth separately using a cross-fostering experiment. Through the cross-fostering design I was able to separate the effects of inbreeding on egg production and subsequent offspring care (incubation and chick rearing). For hatching success, there were moderate declines with both inbreeding in the egg laying mother and inbreeding in the incubating mother, however, in neither case was this statistically significant. Hatchling mass was influenced by interactive effects of clutch size and inbreeding in the egg laying mother, however, this effects was also mediated by the experimental replicate in which the females laid. Post-hatching survival showed a decline in chicks of inbred biological mothers, however the effect of inbreeding in the biological mother was attenuated in larger broods. Although there were not clear cut effects of maternal inbreeding on offspring viability in either stage (egg production or incubation), the cumulative effects of maternal inbreeding across all stages of offspring development could be biologically significant (Frankham *et al*. 2002). Overall, hatching success declined by 18% in offspring where both the biological and foster mother were inbred compared to offspring with control biological and foster mothers. This figure rises to a decline of 45% for survival from egg to 35 days (independence).

5.2 The Magnitude of Inbreeding Depression

Inbreeding depression is predicted to be high for life-history traits as these traits represent wide mutational targets due to the large number of loci influencing them (Rowe and Houle 1996; Merila and Sheldon 1999). A review by DeRose and Roff (1999) found weak inbreeding depression in morphological traits (i.e. adult body size) of 2.2% and relatively strong inbreeding depression for life-history traits of 11.8%. I found inbreeding depressions of 8.3% for yolk mass and 8.5% for incubation attentiveness. In addition resting VO_2 and lean central organ mass (corrected for body mass) were elevated by 13.4% and 21.2% in inbred females respectively. Maternal inbreeding in both the egg laying mother and the foster mother also showed moderate to high levels of inbreeding depression, although due to low statistical power, did not always show statistically significant differences. For inbreeding in the egg laying mother, hatching success declined

by 8.9% (although not statistically significantly), hatching mass by 4.1%, (although mediated by both clutch size and experimental replicate) and post-hatching survival by 4.3% (mediated by the effect of hatching position). For inbreeding in the incubating mother, hatching success declined by 8.5% (although not statistically significantly). My results therefore suggest that inbreeding can have high impacts on traits closely related to fitness. As mentioned above (section 5.1) the effects of maternal inbreeding may have a cumulative effect on survival. Firstly, inbreeding depression can act cumulatively through the combined effects of inbreeding in the egg laying and foster mother; hatching success showed a decline of 8.9% and 8.5% for inbreeding in the egg laying and foster mother respectively but an 18% decline if both effects are taken into account. Secondly, maternal inbreeding can act cumulatively throughout the developmental period. The effect of maternal inbreeding on offspring survival rose from an 18% decline in survival at hatching to a 45% decline in survival by day 35. I did not measure the effects of maternal inbreeding beyond this point but the effects of reduced maternal investment observed in chapters two and three could potentially affect offspring far beyond early survival (Krist 2011; Gorman *et al.* 2004).

5.3 Metabolic Traits and Inbreeding Depression

I found that the resting levels of oxygen consumption (VO_2) were higher in inbred females compared to control females (when controlling for body mass). If resting metabolic rate, but not maximum metabolic rate is increased by inbreeding this could lead to a decrease in the metabolic scope (the difference between resting metabolic rate and maximum metabolic rate) in inbred individuals. The reduced levels of parental care seen in inbred females could therefore potentially be caused by a reduction in energy availability for activities other than self-maintenance. Since resting metabolic rate has been found to correlate with a wide number of fitness traits such as survival (Larivée *et al.* 2010; Artacho & Nespolo 2007; Jackson *et al.* 2001) and reproductive success (Nilsson & Råberg 2001; Blackmer *et al.* 2005) this result could potentially have far reaching implications on the fitness of inbred animals. Increased resting metabolic rate in inbred birds could result from reduced metabolic efficiency, for example, from increased mitochondrial protein leak (Rolfe & Brand 1997; White & Kearney 2013). Increased metabolic rate with either inbreeding (Richardson *et al.* 2004; Ketola & Kotiaho 2009a) or decreased heterozygosity (Garton *et al.* 1984; Rodhouse & Gaffney 1984; Mitton *et al.* 1986; Danzmann *et al.* 1987) has been found in previous studies and may be caused by the cumulative effect of small

mutations affecting, for example mitochondrial function. Because of its wide reaching implications for life-history traits in animals, the effect of inbreeding on metabolic rate is an exciting new avenue of research for the study of inbreeding depression.

5.4 Future Directions

This thesis has shed light on the underlying causes of maternal inbreeding depression. The most promising lines of evidence suggest that reduced offspring survival of inbred mothers may be caused by reduce maternal investment. It would therefore be interesting to study the effects of inbreeding on egg production, and its implications, in a more natural setting. The challenge of study maternal inbreeding in the wild will be obtaining a large enough number of highly inbred individuals to give adequate statistical power. This is an exciting prospect as few studies have found a link between inbreeding and egg production and the observed patterns of egg quality and incubation attentiveness are novel insights. Reduced egg mass is predicted to lead to reduced hatching success, hatching mass and survival of offspring (reviewed in Krist 2011). Reduced incubation attentiveness has also been associated with reduced offspring viability (e.g. Gorman & Nager 2004; Cook *et al.* 2005; Gorman *et al.* 2005a; 2005b). Maternal inbreeding could therefore affect offspring viability through effects on both egg production (by reducing the resources available to the developing embryo) and incubation attentiveness (through reduction in thermal constancy for developing embryos). This could help to explain the finding that inbred mothers often show reduced hatching success (e.g. van Noordwijk & Scharloo 1981; Keller 1998; Marr *et al.* 2006; Cordero *et al.* 2004; Su *et al.* 1996) or reduced offspring survival (e.g. Jamieson *et al.* 2003; Richardson *et al.* 2004; Brouwer *et al.* 2007). It would also be interesting to see if the reduction in maternal incubation attentiveness shows the same decline in wild birds and whether this is compensated for by the partners of inbred females and if maternal incubation attentiveness declines in mono-parental species, where there would be no opportunity for male compensation. If these declines do occur in wild birds there are many important consequences that could be investigated as incubation effort has been found to affect both immediate and future fitness prospects for offspring (e.g. Gorman & Nager 2004; Gorman *et al.* 2005). In light of the findings showing higher resting metabolic rate in inbred females compared to control females, research into the metabolic costs of inbreeding and its effects on parental care would also be an interesting line of research.

Since inbreeding depression is normally attenuated under benign conditions (reviewed in Armbruster & Reed 2005) these effects are expected to be, if anything, stronger in the wild. Another interesting avenue of research might be to vary conditions in the laboratory, such as increasing foraging effort, or giving reduced or unpredictable food supplies to study the inbreeding-environment interactions under laboratory conditions. In my own experiments inbreeding depression often varied according to other factors such as clutch size, hatching order and replicate which may suggest condition dependence of inbreeding depression in many traits.

5.5 Conclusions

This thesis has shed light on the underlying causes of maternal inbreeding depression. My studies suggest a role of reduced allocation of resources to parental care in inbred females, despite taking place under benign laboratory conditions. This reduction in parental care could affect the fitness of offspring from inbred mothers. Future work could examine the fitness consequences of these declines in parental care in wild populations, and the inbreeding-environment interactions of maternal inbreeding depression, to judge the generality of these effects. My finding that maintenance metabolism is increased in inbred females compared to control females could suggest that changes in metabolism may in part underlie the reduction in maternal investment of inbred females. Future studies should focus on the relationship between inbreeding, metabolic scope and parental investment. Inbreeding depression has been found to have important impacts on fitness of natural populations (Crnokrak & Roff 1999). A study by Smith & Bernatchez (2007) estimated that animal and plant populations and their habitats are declining at a rate of 1% per annum. Population declined and fragmentation are likely to results in a higher instance of inbreeding in wild populations. Consequently, research on inbreeding depression has never been so relevant.

Appendix One: Details of sample sizes and dates of experiment

Table 6.1 Inbred and control birds were produced by pairing brother sister pairs and control pairs of zebra finches in December 2008. A random selection of 32 inbred and 32 control females produced from of these pairs were then bred in two replicates in July (inbred, n=16, control n=16) and October 2009 (inbred, n=16, control n=16). The first clutch was removed in order to examine the effects of maternal inbreeding on egg production. Females were immediately allowed to lay a 2nd clutch in order to study the effects of inbreeding on incubation behaviour and offspring viability. In July 2011 the females that had survived from the original 64 experimental females were measured for resting metabolic rate. Finally in April 2012 the females that survived from the original 64 were euthanized by dislocation of the vertebrae under home office regulations and central and peripheral organ mass were examined

n=number of experimental females tested (number paired).	**December 2008-May 2009**	**July/October 2009**			**July 2011**	**April 2012**
	Breeding F1 generation	**32 inbred and 32 control females were paired with unrelated control males over two replicates**			**Resting metabolic rate of inbred and control females measured (chapter 4)**	**Birds euthanized and organ mass of inbred and control females measured (chapter 4)**
		First clutch	**Second clutch**			
		Egg production (chapter 2)	**Incubation behaviour (chapter 3)**	**Hatching success/survival (chapters 2 and 3)**		
Brother x sister	n=17 (19)	n=23 (32)	n=14 (32)	n=14 (32)	n=22 (32)	n=13 (32)
Control	n=16 (21)	n=26 (32)	n=15 (32)	n=15 (32)	n=19 (32)	n=14 (32)

Appendix Two: Molecular Sex Determination

DNA extraction

DNA was extracted using DNeasy Blood & Tissue Kit (Quigen) as per manufactures instructions. A small amount of tissue (approximately $2mm^3$) was taken from each tissue sample and added to an Eppendorf tube containing a mix of 20µl of protease K and 180µl of buffer ATL. Samples were incubated and shaken overnight at 55ºC. The next day the samples were allowed to return to room temperature and then gently vortexed for 5 seconds per sample. Buffer AL (200µl) was added to each sample before vortexing again for 5 seconds. Ethanol (200µl) was then added to each sample before a final vortex for 5 seconds. The mixture was then transferred to spin columns and centrifuged at 8000rpm for 1 minute. Flow through was discarded and the remaining sample was added to 500µl of buffer AW1 and centrifuged at 8000rpm for 1 minute. Flow through was discarded and the column was placed in a new 2ml collection tube before 500µl of buffer AW2 was added and the samples centrifuged at 14,000 rpm for 3 minutes. Flow through was discarded and the column placed in a new 2ml micro-centrifuge tube, 20µl of AE elution buffer was added to the membrane tubes and left for 2-3 minutes. Tubes were then centrifuged at 8000rpm for 1 minute. Another 20µl of AE buffer was then added to each tube and left for 2-3 minutes before a final centrifuge at 6000rpm for 1 minute.

Polymerase Chain Reaction

In birds, sex is genetically determined by the sex chromosomes Z and W. Males are homozygous (ZZ) while females are heterozygous (ZW). Sex was determined using amplification of the Chromodomainhelicase-DNA-binding protein (CHD) genes using polymerase chain reaction (PCR) using primers P2 (5'- tctgcatcgctaaatccttt -3') and P17 (5'- agaaaatcaattccagaagtcca -3'). The CHD gene is located on the W (CHD-W) and Z chromosomes (CHD-Z). Although the exons (expressed regions) of this gene are highly conserved, the introns (unexpressed regions) vary in size between W and Z chromosomes. Therefore the amplified regions of the CHD gene from Z and W chromosomes can be distinguished using gel electrophoresis. The reaction is preferential for CHD-W and so avoids incorrect assignment of sex caused by one gene failing to amplify.

PCR master mix containing 0.8µl each of P2 and P17 primers (concentration of 100ng/µl each), 0.8µl dNTPs (0.2mM for each nucleotide, Promega), 2µl 5X Green GoTaq Flexi Buffer (Promega), 0.8µl MgCl (25mM), 0.075µl GoTaq Flexi DNA polymerase (5u/µl, Promega) and 3.725µl H_2O and 1µl of DNA template.

PCR temperature profile:

Step 1: DNA denatures at 94°C (2 minutes)
Step 2: DNA denatures at 94°C (30 seconds)
Step 3: Primers anneal at 49°C (45 seconds)
Step 4: DNA elongation 72°C (45 seconds)
Steps 2-4 repeated for 30 cycles
Step 5: Final annealing temperature at 49°C (1 minute)
Step 6: Final extension temperature at 72°C (5 minutes)
Step 7: Holding temperature at 12°C

Gel Electrophoresis

After DNA amplification samples were run with gel electrophoresis (Hybaid electro 4 electrophoresis system) through a 3% agarose gel containg 0.2µg/ml ethidium bromide immersed in 1xTBE buffer. The fragment of CHD-W amplified has lower molecular weight than the fragment of amplified CHD-Z and so moves faster through the gel. Female DNA samples are characterised by two bands while male DNA samples are characterised by only one band. DNA samples were mixed with loading buffer (5x Green GoTaq Flexi Buffer, Promega) and added to individual wells of the gel. In addition to the DNA samples, three control samples; one blank with no DNA, one female positive control (ZW) and one male positive control (ZZ) were added to each gel along with a kilobase ladder (Invitrogen). The gel was run at 100V for 2 hours after which point DNA samples were identified by visual examination using a UV light box with reference to the male and female control samples.

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