Disentangling genetic and prenatal maternal effects on offspring size and survival

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Abstract

Organisational processes during prenatal development can have long-term effects on an individual's phenotype. Because these early developmental stages are sensitive to environmental influences, mothers are in a unique position to alter their offspring's phenotype by differentially allocating resource to their developing young. However, such prenatal maternal effects are difficult to disentangle from other forms of parental care, additive genetic effects and/or other forms of maternal inheritance, hampering our understanding of their evolutionary consequences. Here we used divergent selection lines for high and low prenatal maternal investment, and their reciprocal line crosses, in a precocial bird, the Japanese quail (*Coturnix japonica*), to quantify the relative importance of genes and prenatal maternal effects in shaping offspring phenotype. Maternal, but not paternal, origin strongly affected offspring body size and survival throughout development. Although the effects of maternal egg investment faded over time, they were large at key life stages. Additionally, there was evidence for other forms of maternal inheritance affecting offspring phenotype at later stages of development. Our study is among the first to successfully disentangle prenatal maternal effects from all other sources of confounding variation and highlights the important role of prenatal maternal provisioning in shaping offspring traits closely linked to fitness.

Introduction

A considerable amount of variation in an individual's morphology, behaviour, physiology and reproductive performance is influenced by irreversible, organisational processes occurring during prenatal development (Henry and Ulijaszek 1996). Consequently, the environment in which this early development occurs has a significant impact on an individual's phenotype (Clark and Galef 1995; Lindström 1999; Rhind et al. 2001). In most taxa, this prenatal environment is provided by mothers allowing them to alter the developmental trajectory of their offspring through differential allocation of resources and developmental cues (i.e. 'maternal effects'; Mousseau and Fox 1998; Wolf and Wade 2009). Such maternal effects can have far reaching impacts, such as altering the response of offspring traits to selection (Kirkpatrick and Lande 1989) and influencing population dynamics (Ginzburg 1998). However, despite their importance, prenatal maternal effects have proved difficult to isolate from both additive genetic effects and the broader spectrum of maternal inheritance.

As mothers provide their offspring with both genes and the developmental environment, maternal effects are prone to be confounded with direct additive genetic effects. Although exchanging offspring between families ('cross-fostering') can separate postnatal maternal effects from both prenatal and direct genetic effects, this experimental technique cannot remove the potential covariance between prenatal maternal effects and offspring genotype (additive genetic correlation), as cross-fostering would have to happen before prenatal maternal effects occur (Krist and Remeš 2004; Tschirren and Postma 2010; Wolf et al. 2011). The use of isogenic or inbred lines, along with an environmental manipulation would also allow additive genetic effects to be removed (e.g. Donohue et al. 2005), although in the majority of systems such an experiment would not be feasible. Due to the difficultly of isolating prenatal maternal effects, they are often assumed to be negligible (e.g. McAdam et al. 2002), despite potentially having equal or even larger effects on offspring than postnatal effects (Wolf et al. 2011). From an evolutionary perspective, it is crucial to distinguish between additive genetic correlations and prenatal maternal effects

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as the two processes lead to different evolutionary dynamics, including (in the case of maternal effects) an increased responsiveness to selection and the presence of evolutionary momentum (Kirkpatrick and Lande 1989).

Furthermore, there are several additional sources of resemblance between mother and offspring, including mitochondrial inheritance and, in taxa with a ZW sex-determination system, W-linkage, all of which we will refer to by the term 'maternal inheritance'. As with additive genetic effects, maternal effects may be confounded by a genetic correlation between maternal and offspring traits via maternally inherited genes (maternal genetic correlation). These different pathways of maternal inheritance can lead to different evolutionary dynamics (Wolf and Wade 2009). Yet, in most systems, it is difficult, if not impossible, to distinguish between them, and to date the majority of studies have been unable to do so.

In oviparous species, prenatal development occurs outside of the mother's body in a seed or egg. This provides an ideal model for the study of prenatal maternal effects as these units are provisioned before the zygote has formed, preventing the confounding effect of offspring genes acting on maternal investment (e.g. through the placenta in mammals; Moore 2012). In oviparous species, propagule size is often taken as a measure of the quantity of per offspring prenatal maternal investment (Bernardo 1996; Williams 1994). However, the role of egg size in mediating maternal effects has been the matter of debate (Bernardo 1996; Williams 1994, 2012), mainly because the plethora of correlative studies that report a relationship between egg size and offspring quality traits (Fox and Czesak 2000; Krist 2011) are unable to disentangle prenatal maternal effects from direct genetic effects or other forms of inheritance (Krist and Remeš 2004; Sinervo 1993*a*).

A direct manipulation of egg size would best resolve these issues (Krist and Remeš 2004; Sinervo 1993*b*). However, despite success in some taxa (e.g. in lizards; Sinervo and Licht 1991*a,b* and sea urchins; Sinervo and McEdward 1988), this is generally difficult to achieve without a negative impact on the developing embryo (Finkler et al. 1998; Morley et al. 1999). Moreover, manipulations typically involve a simulated reduction in egg size and so an *a priori* prediction of negative

consequences to the offspring (Finkler et al. 1998; Morley et al. 1999; Sinervo and McEdward 1988; but see Sinervo and Licht 1991*a,b*). It is therefore difficult to disentangle the consequences of reduced egg size from the negative effect of the manipulation itself. Furthermore, indirect manipulations of egg size, for example through a manipulation of the mother's hormonal state, are likely to affect other aspects of female physiology, behaviour and/or egg composition (e.g. Wagner and Williams 2007; Williams 2000, 2001). Thus, somewhat surprisingly, direct evidence for a causal effect of maternal egg investment on offspring phenotype is still lacking in most taxa.

In this study, we address these longstanding issues using artificial selection for divergent maternal egg investment in a precocial bird, the Japanese quail (*Coturnix japonica*). To separate the effects of maternal egg investment from additive genetic correlations between maternal and offspring traits, we reciprocally crossed these divergent lines to produce 'hybrid' chicks that have similar genotypes (i.e. one parent from the high investment line and one parent from the low investment line), but differ with respect to the amount of resources they received from their mother before hatching (i.e. depending on their maternal line). By comparing pure-bred and hybrid half-siblings this experimental approach allowed us to separate maternal inheritance from correlated additive genetic effects (Reznick 1981). To further disentangle maternal effects from other forms of maternal inheritance, and to estimate the relative importance of egg size in mediating maternal effects on offspring size, we used a number of complementary statistical approaches. Our results show that there was a strong effect of maternal but not paternal line on offspring phenotype throughout development, persisting until adulthood. Correspondingly, there was a large effect of maternal identity on offspring size and mass, and this maternal variance was entirely explained by egg size. Additionally, we found evidence of other forms of maternal inheritance at later stages of development, which have previously not been described. This study, therefore, unequivocally demonstrates that prenatal maternal provisioning can have strong effects on offspring traits closely linked to fitness at key life stages.

Methods

Selection lines

The Japanese quail study population was kept in large outdoor aviaries on the campus of the University of Zurich, Switzerland. Virgin females were kept in a single sex aviary and males in a mixed sex aviary (5.5 x 7m each) together with females that had previously bred. For this study we used established selection lines for high and low prenatal maternal investment. A detailed description of the selection procedures and responses to selection are presented in Pick et al. (2016). In brief, we selected for high and low relative egg size (i.e. egg size corrected for female body size), by incubating eggs from the highest and lowest 25% of females from a base population (generation one), creating high investment and low investment lines, respectively. In subsequent generations we selected the most extreme 50% of females within each line. This procedure was repeated twice to create two independent replicates per line. By generation four, the lines differed in absolute egg size by 1.2 standard deviations (high investment line: 12.46 ± 0.94g, low investment line: 11.12 ± 0.91g; Pick et al. 2016).

Breeding Design

If maternal and offspring traits are linked by an additive genetic correlation (i.e. the same genes underlie maternal and offspring traits), artificial selection on maternal investment will result in a correlated response in offspring phenotype. Therefore, comparing differences between the lines alone (analogous to a correlative approach), we cannot distinguish the effects of maternal investment from an additive genetic correlation. By reciprocally crossing the lines, and comparing the resulting pure-bred and hybrids offspring, we can experimentally test whether the correlation between maternal investment and offspring phenotype is due to an additive genetic correlation between maternal and offspring traits or maternal inheritance (*sensu lato*) of the offspring trait (see below for specific predictions).

In order to do this, we bred 80 females and 80 males (20 per sex and line replicate) from

the fourth generation of the selection lines. In order to control matings, male-female pairs were brought into breeding cages (122 x 50 x 50cm) inside our breeding facility (see Pick et al. 2016 for a detailed description of animal husbandry). All individuals were mated twice (in different breeding rounds), once with an individual of the same line, and once with an individual of the other line, resulting in both 'pure-bred' and 'hybrid offspring', respectively, for each parent. The order of the matings (same line vs. different line) was randomized.

The mean egg size of a female did not differ if she mated with a male from her own line or the other line (paired t test; high investment females: $t_{38} = 0.323$, $p = 0.748$; low investment females: $t_{36} = 0.848$, $p = 0.402$), showing that there was no differential allocation in response to male line origin. The pairs were kept in cages for 25 days. Between breeding rounds, individuals spent six to eight weeks in their respective aviaries. Five females (one high investment, four low investment), but no males, died between breeding rounds and one of these females was replaced between the two breeding rounds, leading to a total of 156 pairings.

From each pair 7-11 eggs were collected and incubated under standardised conditions as described in Pick et al. (2016). The hatcher was opened once on day 17 of incubation and again on day 18 to remove hatched chicks, after which the hatcher was switched off and any unhatched embryos euthanized. In total, 1475 eggs were incubated and 912 chicks hatched. At hatching, chicks were marked with individually numbered leg rings, and weighed to the nearest 0.01g. They were then reared in mixed line groups in heated cages (chicks per cage: mean $\pm$ sd: 27 $\pm$ 5; range: 20-37) at 35-38°C for the first five days, followed by a slow progression to 25°C over the next nine days. At the age of two weeks, tarsus length (to the nearest 0.1mm) and body mass (to the nearest 0.01g) were measured and chicks were moved to standard cages where they were kept in mixed line groups (chicks per cage: mean $\pm$ sd: 18 $\pm$ 1; range: 14-19). When they were four weeks old, they were sexed according to their plumage, tarsus length (to the nearest 0.1mm) and body mass (to the nearest 0.1g) were measured and they were moved into the outdoor aviaries. 59 chicks died between hatching and week two post-hatching, and two more between weeks two and four post-hatching. A tissue sample was collected from these chicks for molecular sex

determination following Fridolfsson and Ellegren (1999). Adult tarsus length was measured (to the nearest 0.1mm) in 765 offspring when they reached their final body size (at an age of $>$ five weeks old, by which time the tarsus is fully grown; J.L. Pick, unpublished data).

All procedures were conducted under licenses provided by the Veterinary Office of the Canton of Zurich, Switzerland (permit numbers 195/2010; 14/2014; 156). Collected data have been deposited in the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.40jp4 (Pick et al. 2016)

Statistical analysis

To quantify the effect of differential prenatal maternal investment on offspring phenotype we used a combination of complementary statistical approaches. Firstly, we analysed the effect of the maternal and paternal selection line on chick phenotype, which allows for the separation of maternal inheritance (*sensu lato*) from correlated additive genetic effects (Reznick 1981). Secondly we used a hybrid variance component/trait-based modelling approach to both corroborate the presence of maternal inheritance in offspring size, and to quantify the relative importance of a specific maternal trait, egg size, in mediating these maternal effects (McAdam et al. 2014). Finally we used a within- and between- subject approach to distinguish between egg size effects and correlated forms of maternal inheritance (van de Pol and Wright 2009). This combination of experimental techniques (i.e. selection lines and their crosses) and complementary statistical approaches allowed us to comprehensively isolate the effect of maternal investment on offspring phenotype, as well as identifying other forms of maternal inheritance - something that has not been possible in previous studies.

To analyse the effect of the maternal and paternal selection line on chick phenotype (Approach 1), we used mixed effects models including maternal line, paternal line, offspring sex, replicate and hatching day as fixed factors. The two-way interactions between maternal and paternal lines and maternal line and offspring sex were also included. The former tests for heterosis, whilst the latter tests for W-linkage, as only female offspring inherit the W-chromosome

from their mother. Paternal identity, maternal identity, and the interaction between the two, as well as the common rearing environment (i.e. incubator, chick cage or juvenile cage) were included as random effects (random intercepts).

An effect of the maternal line only would indicate that the offspring trait in question is influenced by maternal egg investment (i.e. offspring are large because they developed in a large egg), or other forms of maternal inheritance (e.g. mitochondria). An equal effect of maternal and paternal line (i.e. the two types of hybrids are intermediate to the two types of purebreds), on the other hand, would indicate that the correlation between egg size and the offspring trait in question is due to an underlying genetic correlation between maternal and offspring traits (i.e. large mothers who lay large eggs also pass on alleles for large size to their offspring), rather than a causal effect of egg investment on the offspring trait. An equal effect of maternal and paternal line would be expected under this scenario because both mother and father carry and transfer these correlated genes to their offspring. Note that a lack of a paternal line effect in these analyses does not mean that there is no additive genetic basis to the offspring trait, but rather that there is no genetic correlation between the additive genes for the offspring trait and egg size. Finally, if both maternal inheritance and correlated additive genetic effects affect offspring traits, we would find a significant effect of both maternal and paternal line, with the maternal line effect being significantly larger than the paternal line effect (i.e. the two types of hybrids are significantly different from each other). In order to distinguish between these hypotheses, we conducted post-hoc tests following (Hothorn et al. 2008). We tested specifically whether the maternal line effect was significantly larger than the paternal line effect, which, in the absence of a significant interaction between the two terms, is equivalent to testing for a difference between the two hybrid groups.

Morphological traits at different ages (body mass at hatching, week two and week four, tarsus length at week two, week four and adulthood) were analysed using linear mixed effects models. All measures were standardised (mean=0, SD=1) to aid in the comparison of the strength of effects between life stages. Survival to two weeks of age was analysed using a generalised linear

mixed effect model with a binomial error distribution and a logit link function including the factors outlined above. As 96% of chick mortality occurred within 5 days of hatching, the two chicks that died between two and four weeks of age were excluded from the analysis because their cause of death likely differed from that of chicks dying shortly after hatching. Furthermore, four chicks died from accidental causes, and so were also excluded from the survival analysis. One chick could not be sexed with molecular methods and was excluded from all analyses. We also directly estimated the effects of egg size on survival, by modelling survival as a function of egg mass in the same model structure, but without maternal and paternal lines.

For all models described above, we performed a backwards stepwise deletion of non-significant terms. The significance of fixed effects was determined through likelihood ratio tests between two nested models, in one of which the factor of interest had been removed. The degrees of freedom in all tests was one. Maternal and paternal line terms were always kept in the models, as were the random effects. Analyses were performed in the R statistical framework (version 3.0.3; R Core Team 2014) using the packages lme4 (version 1.1-6; Bates et al. 2014) and multcomp (version 1.4-1; Hothorn et al. 2008).

Although the analysis described above (Approach 1) allows us to demonstrate the presence of maternal inheritance, it does not allow this effect to be linked to a particular maternal trait (i.e. egg size). In a second step (Approach 2), we therefore used a hybrid variance component/trait-based modelling approach (McAdam et al. 2014), which links maternal variance (V_M) to specific maternal traits. This approach has been advocated in order to reconcile trait-based and variance component approaches for the estimation of maternal effects, but as of yet has not been widely used (McAdam et al. 2014; Noble et al. 2014). To estimate V_M and additive genetic variance (V_A), we ran animal models for body mass and tarsus length at all ages. In short, an animal model is a type of mixed effects model that allows for the direct estimation of V_A by utilising the relatedness of all individuals in a pedigree (in this case among parents, full- and half-sib offspring; Kruuk 2004). Although our half-sib breeding design could also be analysed using a standard mixed effects model fitting maternal and paternal identity as random effects (Lynch and Walsh 1998),

an animal model has the advantage that it allows for the direct estimation of V_A and V_M, as well as their associated error. Additionally the models included a V_M term (maternal identity) and a common rearing environment term (see above) as random effects as well as offspring sex and hatching day as fixed factors (variance component model). In the next step, we ran the same animal models but with egg size included as an additional fixed effect (trait-based model). The decrease in V_M between the two models is a measure of the contribution of egg size to the maternal effect term (McAdam et al. 2014). Both egg size and the trait in question were standardised across the whole population (mean=0, SD=1) prior to analysis, meaning that V_M and V_A are equivalent to m^2 and h^2 (the proportion of variance due to maternal and additive genetic effects i.e. heritability), respectively, and that estimates of the effect of egg size are comparable across traits and ages. As these egg size estimates are in units of SD, they were squared in order to approximate how much variation they explain. Egg size was not centered within-sex as the size of the eggs that male and female offspring originated from was extremely similar (mean $\pm$ SD: Males = 11.95 $\pm$ 1.22g; Females = 12.16 $\pm$ 1.19g).

Although this hybrid variance component/trait-based modelling approach allows us to link an observed maternal effect to a specific maternal trait (i.e. egg size), it does not allow for the full distinction between maternal effects (mediated by maternal egg investment) and other forms of maternal inheritance, as there could be a maternal genetic correlation between egg size and offspring traits via maternally inherited genes (e.g. mitochondrial genes). In a third step, we therefore compared within- and between-mother effects of egg size on offspring phenotype (Approach 3) within the animal model framework outlined above. This approach is difficult to use in most taxa, and especially in wild populations, as an individual offspring often cannot be assigned to an individual egg and so previous studies have lacked power to make such a comparison (e.g. Hadfield et al. 2013). If there is a causal relationship between egg size and chick phenotype, then the strength of the within-mother effect of egg size on offspring phenotype should be the same as the between-mother effect (Hadfield et al. 2013), as variation in egg size within a mother should result in the same effect as that between mothers. However, a significantly larger between- than

within-mother effect would indicate the presence of other maternally inherited components (e.g. mitochondria) that partly underlie the correlation between egg size and the offspring trait in question, as all offspring of a mother inherit the same component. We tested this by firstly fitting a model with centered egg size (around the mean of each mother), and mean maternal egg size as covariates in the variance component model described above. The coefficient of the former estimates the within-mother effect of egg size on offspring traits, and the latter estimates the between-mother effect (van de Pol and Wright 2009). Between subject estimates are known to be potentially biased (Lüdtke et al. 2008), however given the repeatability of egg size (0.86) and the mean number of eggs per mother (10.7), this gives a (minimum) reliability of these estimates of 0.984 (see equation 6 in Lüdtke et al. 2008). A second model was fitted which included raw egg size and mean maternal egg size, the latter variable estimating the difference between within- and between-mother effects (van de Pol and Wright 2009). Both derived variables were calculated using standardised egg size (across the whole population), and so are again comparable between different traits and ages. All animal models were run in ASReml (3.00; Gilmour et al. 2009). The significance of fixed effects was estimated using conditional Wald F statistics.

Results

Maternal vs. paternal line effects

Offspring from high investment line mothers were significantly heavier and larger at all stages of development than offspring from low investment line mothers (table 1; fig. 1). The paternal line, however, had no significant effect on offspring body mass or size (table 1; fig. 1). Furthermore, hybrids whose mother originated from the high investment line were significantly heavier and larger than hybrids whose mother originated from the low investment line (table 2; fig. 1). Together these results demonstrate the presence of maternal inheritance in offspring size and mass, and the absence of a genetic correlation between maternal egg investment and offspring size and mass. The maternal line effect was similar in sons and daughters and independent of the line of

the offspring's father (i.e. no interaction maternal x paternal line; table 1) indicating the absence of both W-linked inheritance and heterosis, respectively.

Offspring whose mother originated from the high investment line had a higher survival probability, but there was no effect of paternal line on offspring survival (table 1; fig. A1A). This maternal line effect was significantly larger than the paternal line effect (table 2). Furthermore, egg size was a strong and significant predictor of offspring survival ($\chi^2 = 28.24$, $p < 0.001$; fig. A1B).

Maternal variance and the role of egg size

There were substantial additive genetic and maternal variance components in offspring size and mass at all stages of development (table B1; fig. 2). V_M was reduced to almost 0 in all models when egg size was introduced into the model (table B1; fig. 2), indicating that egg size was the trait underlying this maternal variance.

Egg size had a significant effect on all measured offspring traits (table 3; fig. 2). At hatching, egg size explained 93% of variation in offspring mass (table B1; fig. 2). The strength of this effect on body mass declined between hatching and two weeks post-hatching by 70%, but levelled off between week two and adulthood in both tarsus length and body mass (table B1; fig. 2). This effect mirrored the declining maternal line effect in the line cross analysis (Approach 1 see above; fig. 1a). At adulthood, egg size still explained 15% of variation in offspring size (table B1; fig. 2).

Within- and between-mother effects of egg size on offspring phenotype

In line with the hypothesis that maternal egg investment affects offspring phenotype, there was a significant within-mother effect of egg size on offspring traits at all ages, persisting until adulthood (table 3; fig. 3). As found with the two other, complementary approaches used to assess the effect of differential prenatal maternal investment on offspring phenotype (see above), this within-mother egg size effect declined between hatching and week two and continued to decline after two weeks post-hatching (table B1; fig. 3).

Until week four post-hatching, the between-mother effect was not significantly larger than the

within-mother effect (table 3; fig. 3), indicating that egg size was the sole mediator of maternal effects at these life stages. At week four and at adulthood, however, the between-mother effect was significantly larger than the within-mother effect (table 3 & B1; fig. 3), indicating that there was an additional maternal factor influencing offspring phenotype, which does not vary amongst siblings from the same mother.

Discussion

Assessing the consequences of variation in prenatal maternal investment for offspring performance has proven difficult due to the potential covariance between maternal investment and maternal (and therefore offspring) genetic quality. This has hampered further progress in our understanding of the role of prenatal maternal effects in the evolutionary process. Here we used artificial selection lines for divergent prenatal maternal investment and their reciprocal crosses to experimentally address this long-standing issue. Our results reveal a clear effect of maternal, but not paternal, line on offspring body mass and size throughout development. This provides unequivocal evidence of maternal inheritance and the lack of a genetic correlation between offspring mass or size and maternal investment.

Our half-sibling breeding design furthermore provides evidence for strong effects of maternal identity on offspring phenotype throughout development and these maternal identity effects were fully explained by egg size (see also Hadfield et al. 2013). Therefore, the maternal contribution to variation in offspring phenotype, the entity on which selection is acting, is substantial. Indeed, we found that maternal egg investment had a strong effect on offspring survival early in life, even under benign captive conditions. Arguably, these effects would be even more pronounced under harsher conditions in the wild.

In addition to maternal effects, we observed substantial additive genetic variance for offspring size and mass at all ages, which is in line with previous work (Postma 2014). Importantly, this finding does not conflict with the lack of a paternal line effect (Approach 1). Rather, it highlights

that both parents pass on genes to their offspring that influence size, but that alleles do not systematically differ between the selection lines, or in other words, there is no genetic correlation between body size and egg size.

A particular strength of our experimental set-up is that, in addition to separating the effects of maternal egg investment and autosomal genes, it also allowed us to quantify the role of additional forms of maternal inheritance by comparing effects of within- and between-mother variation in egg size on offspring phenotype - something that has proven difficult to demonstrate in observational studies. If maternally inherited components, such as mitochondria, underlie the relationship between egg size and offspring phenotype (rather than egg size per se), we would predict to find no within-mother effect of egg size variation on offspring phenotype, as all offspring from a given mother inherit the same mitochondria (Hadfield et al. 2013). However, we found substantial within-mother effects of egg size on offspring phenotype. Although these maternal effects became weaker with increasing offspring age (as also observed in other taxa; e.g. Krist 2011; Lindholm et al. 2006; Wilson and Reale 2006), they were still present at adulthood. As juvenile quail are largely independent from their mother by two weeks of age (Launay et al. 1993; Orcutt and Orcutt 1976), this result shows that prenatal maternal investment continues to shape offspring phenotype well after independence.

Apart from survival early in life, we cannot directly link our measures to fitness. However, in many species, size or mass at independence is a strong predictor of survival and recruitment (Both et al. 1999; Bowen et al. 2015; Dias and Marshall 2010), suggesting that prenatal maternal investment contributes to variation in fitness in the wild. Furthermore, a recent study showed that selection on juvenile size is much stronger than on adult size (Rollinson and Rowe 2015), indicating that prenatal maternal investment contributes to a large amount of variation in offspring phenotype ($>20\%$) at a time when selection is strongest.

After independence (i.e. after two weeks of age), a significantly larger between- than within-mother effect of egg size on offspring phenotype was observed. This indicates that besides maternal resource investment in the eggs, other maternally inherited components contribute to

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variation in offspring phenotype at later life stages. Demonstrating such additional forms of maternal inheritance has not been possible in previous non-experimental (e.g. Krist 2011) or experimental studies (Sinervo and Licht 1991*a,b*). We can exclude the role of sex chromosomes, as in birds females are the heterogametic sex, passing on a W chromosome to their daughters and a Z chromosome to their sons. Covariance between egg size and offspring phenotype mediated by genes on either of these chromosomes would therefore result in a sex-specific response of maternal line, which was not observed. In other taxa, such non-additive effects have been attributed to genomic imprinting (Holman and Kokko 2014), which does however not appear to occur in birds (Frésard et al. 2014). Furthermore, it is unlikely that other egg components (such as yolk hormones or carotenoids) explain this between-mother effect. Given that egg size explains all maternal variation, such egg components would have to be extremely highly correlated with egg size, which is usually not the case (Okuliarova et al. 2011; Tschirren et al. 2009).

Mitochondria, which are inherited along the maternal line, are thus likely to cause the additional between-mother effects on offspring phenotype after independence. Recent work has shown that mitochondrial variation can affect an individual's metabolic rate (Kurbalija Novičić et al. 2015; Tieleman et al. 2009; Tranaha et al. 2011), which in turn has been linked to both faster growth and increased maternal investment (Nilsson and Råberg 2001; Rønning et al. 2009; van der Ziel and Visser 2001). The possibility that maternal inheritance of offspring phenotype occurs through sources other than maternal effects (*sensu stricto*) is commonly overlooked, but is important as alternate forms of inheritance lead to different evolutionary dynamics of offspring traits under selection (Wolf and Wade 2009). Care should thus be taken when attributing variance in offspring phenotype purely to maternal effects, when the data available do not allow separation from other forms of maternal inheritance.

The potential for maternal effects to alter the response of offspring traits to selection has long been recognised (Kirkpatrick and Lande 1989). Positive maternal effects, such as those demonstrated here, accelerate the rate at which offspring traits, such as body size, evolve. Such maternal effects can also have far reaching ecological impacts, such as altering population dy-

namics (Ginzburg 1998) and facilitating both niche divergence (Pfennig and Martin 2009) and range expansion (Badyaev et al. 2002). In species that do not show extended parental care after birth (including the majority of oviparous taxa) maternal investment occurs primarily through prenatal egg (or seed) provisioning. However, despite the ubiquity of prenatal investment in nature and the potential for prenatal effects to shape ecological and evolutionary processes, there has been little quantification of these effects, especially when compared with postnatal parental effects. Typically quantitative genetic approaches treat maternal effects as a 'black box' (known as 'maternal performance'), whereby the maternal effectors themselves are often not identified (McAdam et al. 2014). However, it is crucial to understand which maternal traits are causing these maternal effects in the offspring. Whether the maternal traits have a genetic basis is important for assessing the evolutionary potential of these effects, as only heritable maternal traits can respond to selection (Räsänen and Kruuk 2007; Wolf et al. 1998). Furthermore, it is likely that there are constraints acting on maternal investment (Pick et al. 2016), which will indirectly affect the evolutionary dynamics of the offspring traits. For example, reproductive investment in many taxa is known to be costly (Harshman and Zera 2006). These constraints, however, can only be determined if the maternal effector in question in known. Therefore in order to fully understand the evolutionary potential of a trait, it is important to identify the presence of maternal effects, the trait(s) underlying these effects, and their strength, as we have done here.

Conclusion

Although it is often assumed that prenatal maternal investment affects offspring phenotype, conclusive evidence of this phenomenon is surprisingly scarce and studies are frequently unable to disentangle the effects of maternal investment from other confounding sources of variation. Through an underused experimental approach in the study of prenatal maternal effects, we provide direct evidence for strong and persistent effects of prenatal maternal resource investment on offspring traits closely linked to fitness and demonstrate the occurrence of additional forms of

maternal inheritance at later life stages. Thereby this study contributes to a better understanding of how maternal effects shape the evolutionary process.

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Tables

Table 1: Effect of maternal and paternal line, sex, hatching day and line replicate, on body mass, body size, and survival.

	M. Line		P. Line		Sex		Hatch Day		Replicate		M. : P. Line		M. Line : Sex	
	χ^2	p	χ^2	p	χ^2	p	χ^2	p	χ^2	p	χ^2	p	χ^2	p
Body Mass														
Hatching	45.75	<0.001	1.02	0.314	6.57	0.010	60.90	<0.001	4.27	0.039	0.98	0.323	0.07	0.795
Week 2	17.79	<0.001	0.60	0.440	2.10	0.147	48.68	<0.001	0.03	0.865	2.45	0.118	0.41	0.522
Week 4	23.11	<0.001	3.24	0.072	2.71	0.099	22.60	<0.001	0.32	0.574	0.43	0.514	1.06	0.304
Tarsus Length														
Week 2	20.75	<0.001	0.15	0.703	0.67	0.412	47.03	<0.001	0.01	0.924	2.62	0.105	0.07	0.795
Week 4	21.45	<0.001	0.67	0.413	42.42	<0.001	4.04	0.044	0.35	0.552	0.16	0.690	0.04	0.840
Adult	17.68	<0.001	0.32	0.570	114.88	<0.001	0.08	0.783	0.11	0.737	0.21	0.644	0.68	0.409
Survival														
Week 2	4.01	0.045	0.55	0.459	0.00	0.985	11.50	<0.001	1.64	0.201	0.54	0.463	0.00	0.952

Note: M stands for maternal, P for paternal.

Table 2: Maternal and paternal line effects on offspring mass, size and survival.

	Maternal Line	Paternal Line	Difference	χ^2	P	Mean $\pm$ SD
Body Mass						
Hatching	-1.181 $\pm$ 0.150	0.056 $\pm$ 0.055	-1.236 $\pm$ 0.159	7.76	<0.001	8.37 $\pm$ 0.98
Week 2	-0.533 $\pm$ 0.115	-0.072 $\pm$ 0.093	-0.461 $\pm$ 0.147	3.14	0.002	54.12 $\pm$ 7.41
Week 4	-0.602 $\pm$ 0.112	-0.156 $\pm$ 0.085	-0.446 $\pm$ 0.139	3.21	0.001	137.04 $\pm$ 16.07
Tarsus Length						
Week 2	-0.596 $\pm$ 0.120	-0.031 $\pm$ 0.082	-0.565 $\pm$ 0.144	3.93	<0.001	29.62 $\pm$ 1.54
Week 4	-0.629 $\pm$ 0.124	-0.082 $\pm$ 0.100	-0.547 $\pm$ 0.158	3.47	<0.001	38.43 $\pm$ 1.36
Adult	-0.561 $\pm$ 0.123	-0.058 $\pm$ 0.102	-0.503 $\pm$ 0.159	3.16	0.002	39.50 $\pm$ 1.33
Survival	-0.644 $\pm$ 0.320	0.238 $\pm$ 0.322	-0.882 $\pm$ 0.449	1.96	0.050	-

Note: Parameter estimates ($\pm$ SE) represent values for the low investment line compared to the high investment line. 'Difference' represents the difference between maternal low investment and maternal high investment line hybrids. In all comparison, the degree of freedom is equal to one. Variables were standardised before analysis, meaning that model parameter estimates are in units of SD. To aid interpretation of effect sizes, population level Mean $\pm$ SD are presented, body mass in g, tarsus length in mm.

Table 3: Effects of egg size (including within- and between-mother effects, and the difference between them), hatching day and offspring sex on body mass and tarsus length throughout development.

Model	Egg Size			Within-mother			Between-mother			Difference			Hatch Day			Sex		
	F	DF	P	F	DF	P	F	DF	P	F	DF	P	F	DF	P	F	DF	P
Hatching Mass																		
VC	-	-	-	-	-	-	-	-	-	-	-	-	57.44	799.1	<0.001	5.77	793.8	0.017
Trait	4388.69	214.4	<0.001	-	-	-	-	-	-	-	-	-	249.89	866.3	<0.001	12.19	848.5	<0.001
WB	-	-	-	1860.54	717.4	<0.001	2550.65	73.8	<0.001	0.06	344.3	0.796	249.20	865.2	<0.001	12.16	847.6	<0.001
Week 2 Mass																		
VC	-	-	-	-	-	-	-	-	-	-	-	-	48.07	791.5	<0.001	1.93	766.8	0.168
Trait	125.67	213.3	<0.001	-	-	-	-	-	-	-	-	-	61.46	809.7	<0.001	2.50	782.1	0.116
WB	-	-	-	38.35	818.9	<0.001	88.40	119.3	<0.001	0.02	621.9	0.890	61.40	808.5	<0.001	2.50	780.9	0.117
Week 4 Mass																		
VC	-	-	-	-	-	-	-	-	-	-	-	-	24.15	772.2	<0.001	3.26	745	0.074
Trait	96.33	215.1	<0.001	-	-	-	-	-	-	-	-	-	29.65	790	<0.001	3.07	759.8	0.082
WB	-	-	-	11.95	794.7	<0.001	93.20	117.5	<0.001	5.69	602.7	0.018	28.85	790.6	<0.001	3.03	760.3	0.084
Week 2 Tarsus Length																		
VC	-	-	-	-	-	-	-	-	-	-	-	-	47.34	801.6	<0.001	0.78	778.2	0.379
Trait	149.99	115	<0.001	-	-	-	-	-	-	-	-	-	60.83	824.7	<0.001	0.52	796.1	0.470
WB	-	-	-	42.87	774.4	<0.001	108.37	62.5	<0.001	0.07	549	0.778	60.85	823.5	<0.001	0.53	794.7	0.467
Week 4 Tarsus Length																		
VC	-	-	-	-	-	-	-	-	-	-	-	-	3.68	778.1	0.057	45.01	752.9	<0.001
Trait	89.57	237	<0.001	-	-	-	-	-	-	-	-	-	5.85	799.4	0.017	44.17	768.4	<0.001
WB	-	-	-	12.73	813.1	<0.001	85.69	115	<0.001	6.40	549.8	0.012	5.51	800	0.020	44.19	769.3	<0.001
Adult Tarsus Length																		
VC	-	-	-	-	-	-	-	-	-	-	-	-	0.14	690.2	0.699	124.51	675.3	<0.001
Trait	67.13	248.8	<0.001	-	-	-	-	-	-	-	-	-	0.04	704.5	0.843	121.38	684.4	<0.001
WB	-	-	-	7.93	730.8	0.005	68.84	118.5	<0.001	7.09	518.6	0.008	0.01	706.5	0.919	122.26	687.1	<0.001

Note: Three models were run for each trait. The first (VC) contained only hatching day and offspring sex. The second model (Trait) additionally included egg size. The third model (WB) separated within- and additional between-mother egg size effects, through additionally including mean maternal egg size, to estimate additional between-mother effects.
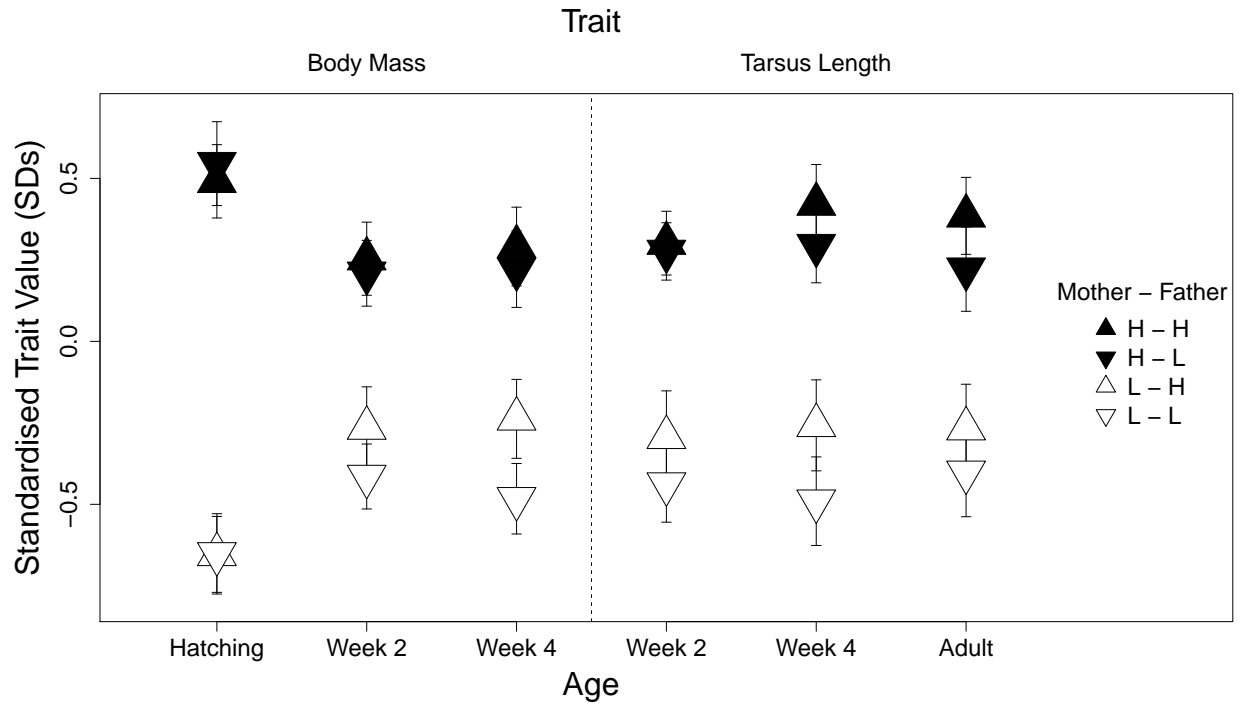
Figures

Figure 1: Body mass and tarsus length throughout development of pure-bred and hybrid offspring from reciprocal crosses of the high and low maternal investment lines. Mean (±SE) of within-pair means are shown. H is an abbreviation of high investment line and L of low investment line. Measures were standardised, meaning that the y-axis is in units of standard deviation and traits are directly comparable.
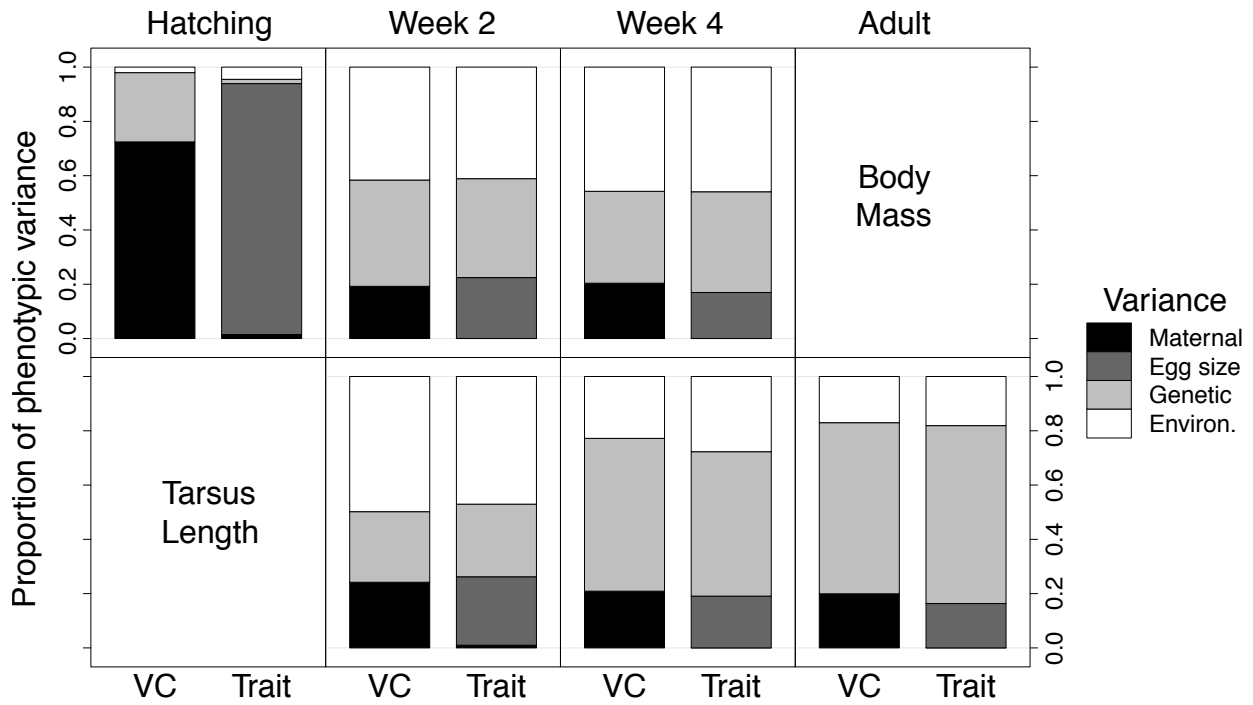
Figure 2: Variance components of body mass and tarsus length throughout development estimated using two animal models. The variance component model (VC) estimates additive genetic variance (V_A), maternal variance (V_M) and environmental variance (V_E, residual and common environmental variance combined) of offspring phenotype. The trait-based model (Trait) additionally includes egg size (dark gray) as a covariate. The output of all models has been scaled to a total variance of 1.

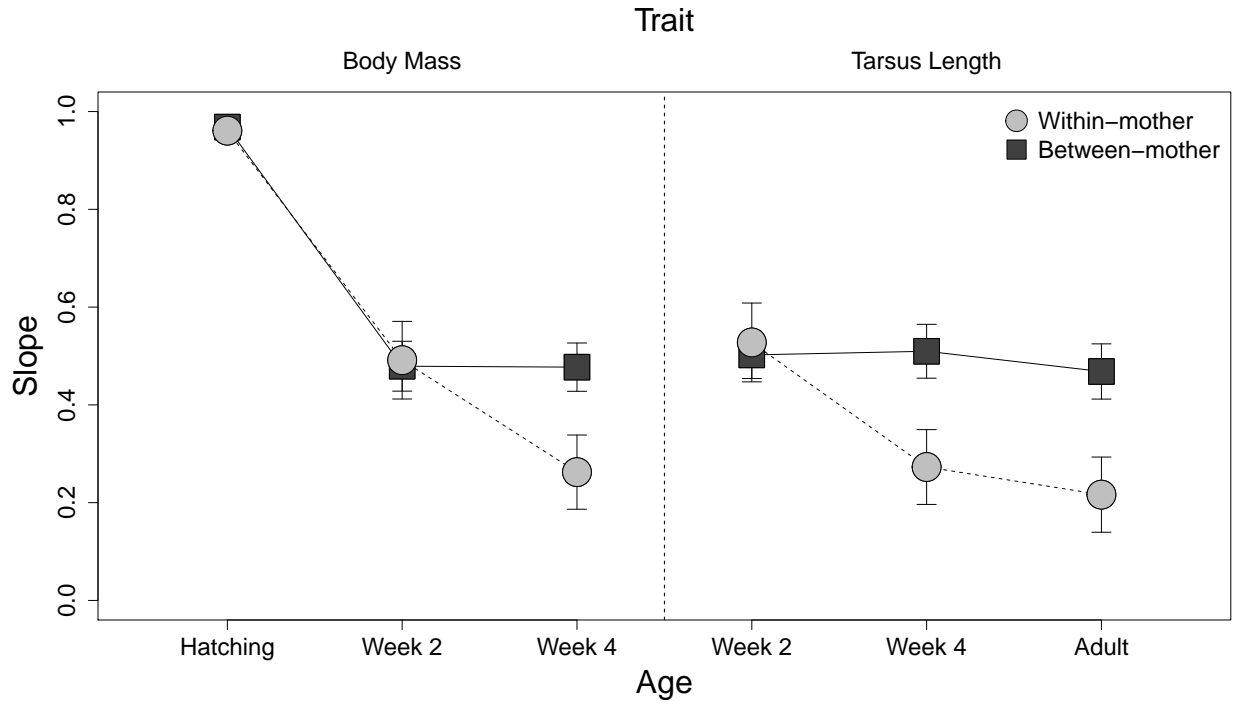Figure 3: Within- and between-mother effects of egg size on offspring phenotype. Parameter estimates $\pm$ SE are shown. Measures were standardised, meaning that the y-axis is in units of standard deviation and traits are directly comparable.
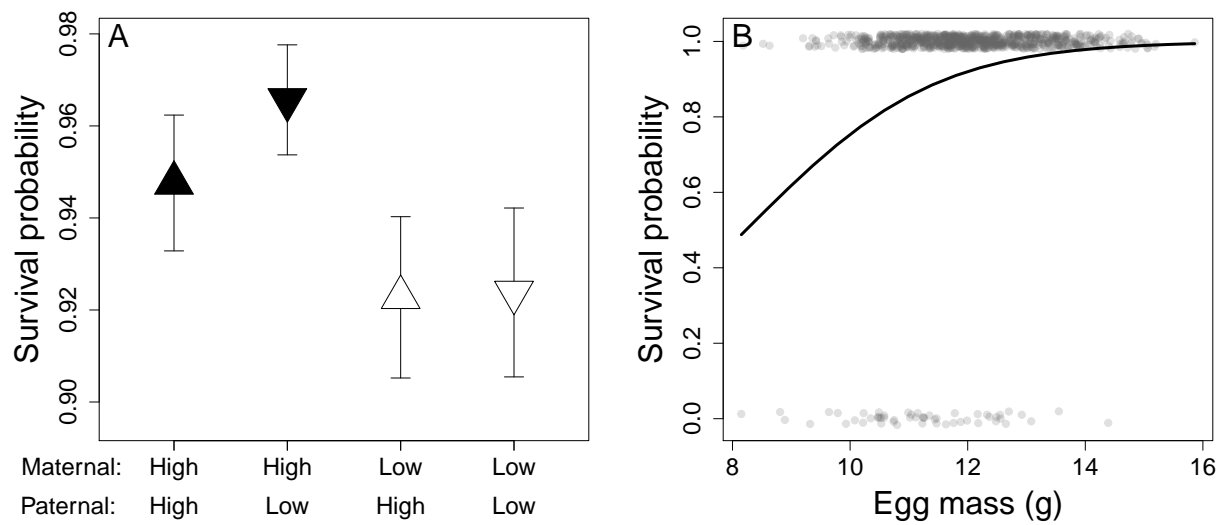
Online Appendix A: Supplementary Figures

Figure A1: Effect of A) maternal and paternal selection line and B) egg mass on offspring survival probability. In A) means ± SE are shown.

Online Appendix B: Supplementary Tables

Table B1: Estimates of fixed effects and variance components from animal models for offspring body mass and tarsus length throughout development.

Model	Fixed Effects						Variance Components			
	Intercept	ES (Raw)	ES (Mean)	ES (Centered)	Hatch Day (18)	Sex (Male)	V_A	V_M	V_E	V_R
Hatching Mass										
VC	0.019 ± 0.109	-	-	-	-0.320 ± 0.042	-0.067 ± 0.028	0.268 ± 0.070	0.759 ± 0.138	0.003 ± 0.003	0.019 ± 0.037
Trait	0.072 ± 0.024	0.965 ± 0.015	-	-	-0.379 ± 0.024	-0.056 ± 0.016	0.016 ± 0.008	0.015 ± 0.005	0.001 ± 0.001	0.044 ± 0.005
WB1	0.073 ± 0.024	-	0.968 ± 0.019	0.961 ± 0.022	-0.379 ± 0.024	-0.056 ± 0.016	0.016 ± 0.008	0.015 ± 0.005	0.001 ± 0.001	-
WB2	0.073 ± 0.024	0.961 ± 0.022	0.007 ± 0.029	-	-0.379 ± 0.024	-0.056 ± 0.016	0.016 ± 0.008	0.015 ± 0.005	0.001 ± 0.001	0.044 ± 0.005
Week 2 Mass										
VC	-0.026 ± 0.093	-	-	-	-0.602 ± 0.087	0.078 ± 0.056	0.403 ± 0.131	0.198 ± 0.074	0.060 ± 0.022	0.368 ± 0.075
Trait	-0.012 ± 0.080	0.483 ± 0.043	-	-	-0.658 ± 0.084	0.086 ± 0.054	0.378 ± 0.076	0.000 ± 0.000	0.076 ± 0.026	0.351 ± 0.051
WB1	-0.012 ± 0.080	-	0.479 ± 0.051	0.491 ± 0.079	-0.658 ± 0.084	0.085 ± 0.054	0.380 ± 0.076	0.000 ± 0.000	0.076 ± 0.026	-
WB2	-0.012 ± 0.080	0.491 ± 0.079	-0.012 ± 0.094	-	-0.658 ± 0.084	0.085 ± 0.054	0.380 ± 0.076	0.000 ± 0.000	0.076 ± 0.026	0.351 ± 0.051
Week 4 Mass										
VC	0.050 ± 0.097	-	-	-	-0.404 ± 0.082	-0.095 ± 0.052	0.347 ± 0.117	0.209 ± 0.070	0.152 ± 0.038	0.317 ± 0.067
Trait	0.058 ± 0.085	0.413 ± 0.042	-	-	-0.440 ± 0.081	-0.090 ± 0.052	0.374 ± 0.074	0.000 ± 0.000	0.167 ± 0.041	0.296 ± 0.048
WB1	0.056 ± 0.084	-	0.477 ± 0.049	0.262 ± 0.076	-0.433 ± 0.081	-0.090 ± 0.052	0.359 ± 0.072	0.000 ± 0.000	0.165 ± 0.040	-
WB2	0.056 ± 0.084	0.262 ± 0.076	0.215 ± 0.090	-	-0.433 ± 0.081	-0.090 ± 0.052	0.359 ± 0.072	0.000 ± 0.000	0.165 ± 0.040	0.302 ± 0.048
Week 2 Tarsus Length										
VC	0.063 ± 0.087	-	-	-	-0.611 ± 0.089	-0.051 ± 0.057	0.265 ± 0.111	0.246 ± 0.073	0.030 ± 0.015	0.477 ± 0.068
Trait	0.071 ± 0.071	0.509 ± 0.042	-	-	-0.667 ± 0.086	-0.040 ± 0.055	0.275 ± 0.100	0.010 ± 0.041	0.047 ± 0.018	0.436 ± 0.061
WB1	0.071 ± 0.071	-	0.502 ± 0.048	0.528 ± 0.081	-0.667 ± 0.086	-0.040 ± 0.055	0.277 ± 0.100	0.010 ± 0.041	0.047 ± 0.019	-
WB2	0.071 ± 0.071	0.528 ± 0.081	-0.026 ± 0.094	-	-0.667 ± 0.086	-0.040 ± 0.055	0.277 ± 0.100	0.010 ± 0.041	0.047 ± 0.019	0.435 ± 0.061
Week 4 Tarsus Length										
VC	0.195 ± 0.091	-	-	-	-0.157 ± 0.082	-0.353 ± 0.053	0.577 ± 0.167	0.214 ± 0.087	0.021 ± 0.011	0.212 ± 0.091
Trait	0.200 ± 0.073	0.429 ± 0.045	-	-	-0.195 ± 0.081	-0.346 ± 0.052	0.513 ± 0.095	0.000 ± 0.000	0.029 ± 0.013	0.238 ± 0.058
WB1	0.198 ± 0.072	-	0.510 ± 0.055	0.273 ± 0.076	-0.189 ± 0.081	-0.345 ± 0.052	0.499 ± 0.093	0.000 ± 0.000	0.027 ± 0.012	-
WB2	0.198 ± 0.072	0.273 ± 0.076	0.237 ± 0.094	-	-0.189 ± 0.081	-0.345 ± 0.052	0.499 ± 0.093	0.000 ± 0.000	0.027 ± 0.012	0.244 ± 0.057
Adult Tarsus Length										
VC	0.270 ± 0.088	-	-	-	0.030 ± 0.079	-0.584 ± 0.052	0.585 ± 0.173	0.185 ± 0.086	0.009 ± 0.009	0.149 ± 0.093
Trait	0.278 ± 0.073	0.380 ± 0.046	-	-	-0.015 ± 0.078	-0.571 ± 0.052	0.578 ± 0.102	0.000 ± 0.000	0.014 ± 0.009	0.146 ± 0.060
WB1	0.278 ± 0.072	-	0.468 ± 0.056	0.216 ± 0.077	-0.008 ± 0.078	-0.572 ± 0.052	0.554 ± 0.098	0.000 ± 0.000	0.012 ± 0.009	-
WB2	0.278 ± 0.072	0.216 ± 0.077	0.252 ± 0.095	-	-0.008 ± 0.078	-0.572 ± 0.052	0.554 ± 0.098	0.000 ± 0.000	0.012 ± 0.009	0.157 ± 0.058

Note: Four models were run for each trait. The first (VC) estimated the variance due to additive genetic (V_A) and maternal identity effect(V_M; V_E refers to the common environment effect and V_R the residual variance). The second model (Trait) estimated the proportion of maternal variance that is explained by egg size (ES), through the decrease in maternal variance between VC and Trait models. The third and fourth models (WB1 and WB2) separate within- and between-mother effects of egg size. In WB1, ES (Mean) estimates between-mother effects and ES (Center) within-mother effects. In WB2 ES (Mean) estimates the difference between within and between mother effects. In all models the offspring trait was standardised. ES (raw) was also standardised, and the other ES measures were derived from standardised ES (raw); see text.