Transgenerational effects of the social environment in Japanese quail, *Coturnix japonica*

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Summary

The social environment of reproducing females can induce changes in behaviour and physiology, with consequences for reproductive investment. Changes in reproductive investment, in turn, may modify the prenatal environment of the developing offspring and can thereby profoundly shape the offspring's future phenotype. Such prenatal maternal effects may drive adaptive transgenerational plasticity, enabling mothers to prepare offspring for their future environmental conditions and thereby increasing their chances of survival. In the case of such anticipatory maternal effects, offspring that experience conditions that match the conditions predicted by the maternal phenotype are expected to perform better than offspring experiencing mismatching conditions. The maternal and offspring environments are thus expected to have interactive effects on offspring phenotypes. We tested for anticipatory maternal effects in a match/mismatch experiment by investigating the (interactive) effects of one important aspect of the social environment – group size – on maternal and offspring physiology, morphology reproduction and behaviour in a precocial avian species, the Japanese quail (*Coturnix japonica*).

In the parental (P0) generation (chapter 2), the social environment of adult female Japanese quail was manipulated by housing the females in pairs (one female, one male) or groups (three females, one male). In previous studies, increased social density or social challenges have been linked to higher circulating androgen and glucocorticoid levels. Against our predictions, females housed in pairs had significantly higher concentrations of circulating androgens and tended to have higher concentrations of circulating corticosterone than females housed in groups. Although the female's baseline hormone levels were affected by the social environment, we found no indication for effects on the response to endocrinological challenges of the main stress (hypothalamic-pituitary-adrenal) and reproductive (hypothalamic-pituitary-gonadal) axis. Furthermore, the social environment had no effects on female reproduction, suggesting that the effects on female endocrine physiology had little fitness consequences. Counter to our expectations, the social environment did not affect yolk testosterone levels, and we did not find a correlation between yolk testosterone levels and the females' response to gonadotrophin releasing hormone (GnRH). We propose that our unexpected findings are due to differences in the exposure to males in our social treatments. In pairs, the male copulatory behaviour may have stimulated female circulating hormone levels more strongly than in groups where effects were diluted due to the presence of other females.

Changes in social density have been shown to affect offspring sex ratio in previous studies, and variation in maternal hormone levels around conception have been suggested as a proximate mechanism underlying such effects. High maternal androgens have repeatedly been linked to increased investment in sons, whereas high glucocorticoid levels are usually related to increased investment in daughters. Even though maternal endocrine physiology was affected, we found no evidence for effects of the maternal social environment or maternal circulating androgen and corticosterone concentrations on

offspring sex ratio or sex-specific juvenile survival (chapter 3). The maternal social environment did also not affect juvenile offspring growth and circulating androgen and corticosterone levels. Our negative results might be explained by the lack of effects on egg mass or yolk testosterone levels in the parental generation, since both are important mediators of maternal effects. Furthermore, differences between the type of social stimuli and the timing of changes in the social environment and hormones with respect to the reproductive cycle and meiosis might explain the contrasting results between studies.

F1 adult females were housed under social conditions that either matched or mismatched their maternal social conditions with respect to group size (pairs of two females and groups of four females; chapter 4). This experimental setup allowed us to investigate the interactive effects of the maternal and adult F1 offspring social environments. We found an interaction effect between the maternal and own social environment on F1 female mass, in combination with a significant effect of the F1 social environment on growth. We initially predicted matched offspring to perform better, however, 'mismatched' group-housed daughters from pair-housed mothers turned out to be heavier overall than females from the other combinations of P0-F1 social environments. Our findings thus support the idea that maternal effects may emerge context-dependent, though the adaptive value of this match/mismatch effect remains speculative. Furthermore, in contrast to our findings in the P0 generation, the social environment of the F1 females did not affect their circulating hormone levels, but affected their growth and reproductive investment. F1 females housed in groups grew more than pair-housed females, which resulted in a maternal effect on egg mass, hatching success and F2 offspring mass at hatching (all increased compared to F1 pair-housed females; chapter 4). These effects on F2 hatch mass could have important consequences for their subsequent growth and survival, which should be further investigated in future studies.

The effects of social group size on female physiology, reproduction, and the next generation differed between the P0 and F1 generations. Differences in the sex ratios of the social environments between the P0 and F1 generation could partly explain these effects.
Taken together, our results indicate that the social environment does affect female physiology and reproduction, and may induce maternal effects on the offspring's phenotype in a context-dependent way. However, our results also indicate that different types of social stimuli induce different effects on females and their offspring. Furthermore, the timing of measurements and manipulations of the social environment or female and offspring physiology is likely an important factor explaining why results vary between studies. To gain a better understanding of the underlying mechanisms and the function of maternal effects of the social environment, it is important to establish which social stimuli are most important, and how effects of social stimuli may interact with each other. The studies described in this thesis point towards a number of factors that should be further investigated, in particular the effects of different adult sex ratios on females and their offspring. Moreover, it is important to further investigate what mediates maternal effects and at which time they manifest. This includes studying how resources accumulate in the yolk, and how environmental factors can influence these processes.

Chapter 1

General introduction

1.1. Prenatal maternal effects and phenotypic variation

The behavioural and physiological appearance of an individual is the product of its genetic 'blueprint' and influences of its internal and external environment. Phenotypic changes in response to variation in environmental conditions are considered adaptive, allowing an individual to cope with environmental perturbations (Fusco and Minelli, 2010; Meyers and Bull, 2002; Piersma and Drent, 2003), although maladaptive consequences have also been described (Ghalambor et al., 2007). Environmental influences on phenotypic characteristics can occur throughout life, from early prenatal stages to late adulthood. Moreover, since parents contribute to the prenatal and often early postnatal environment of the developing offspring, any phenotypic changes that occur in parents, especially mothers, can affect the offspring's phenotypic development. Such non-genetic maternal effects (hereafter named 'maternal effects') have received considerable attention, as they might have important evolutionary consequences, affecting fitness across generations (Mousseau and Fox, 1998).

A well-known example of a prenatal maternal effect is the transgenerational induction of defences in waterfleas (*Daphnia cucullata*). In this species, the presence of a predator induces morphological changes: individuals develop 'helmets' which act as a defence against predators (Agrawal et al., 1999). The offspring of those mothers also show increased helmet development at the neonate stage, even if they have not experienced the presence of a predator themselves. Thus, a high predation risk in the maternal environment induces adaptive morphological changes in the offspring, ensuring defence against predators already shortly after birth.

There are many pathways through which non-genetic maternal effects can establish in the offspring. Examples include the transmission of maternal resources such as nutrients and hormones, parental care, the transmission of behavioural aspects through learning. On a molecular level, transgenerational effects are most likely mediated via epigenetic mechanisms (DNA methylation or histone modification) affecting gene expression or the transmission of RNA (Jensen, 2013; Richards, 2006).

The literature has shown a wide range of environmental stimuli to affect maternal phenotypes, with potential consequences for offspring development. Examples include effects of photoperiod (Horton and Stetson, 1992; Mousseau and Dingle, 1991), predation risk (Agrawal et al., 1999; Giesing et al., 2011; Mommer and Bell, 2014), food availability (Giordano et al., 2014; Plaistow et al., 2006), but also the social environment (Guibert et al., 2010; Kaiser and Sachser, 2009, 2005).

Despite the growing body of literature, many questions regarding the ultimate and proximate causes and consequences of maternal effects remain to be answered and findings further evaluated. The aim of this thesis is to investigate transgenerational effects of the social environment, and their potential adaptive benefits, in an avian model, the Japanese quail (*Coturnix japonica*). While the social environment encompasses various factors that can affect female physiology, reproductive investment and offspring

phenotype, which will be expanded upon in this chapter, our studies focus specifically on the influence of social group size.

1.2. Maternal effects of the social environment

The social environment comprises many different stimuli, as individuals can engage in a wide range of intra- and intersexual social interactions, including agonistic, socio-positive and sexual interactions. The frequency and type of social interactions is strongly influenced by factors such as population density or sex ratio, an individual's social rank or reproductive status. The following sections will present an overview of how the social environment may affect female physiology and reproductive investment, with a main focus on avian species. Furthermore, the importance of the resulting maternal effects for offspring phenotypic development is illustrated at the end of this section.

1.2.1. The social environment, female physiology and reproduction

Variation in the frequency and type of social stimuli can profoundly affect an individual's behavioural and physiological phenotype, which can lead to changes in reproductive investment. Social density and increased intraspecific competition, for example, may affect body mass (Asghar Saki et al., 2012; Keeling et al., 2003; Onbaşılar and Aksoy, 2005), with potential consequences for reproduction, as heavier females may be able to invest in more or higher quality offspring (Christians, 2002; Drent and Daan, 1980; Lim et al., 2014; Ronget et al., 2018; Sockman et al., 2006; Verboven et al., 2003). Furthermore, social encounters can induce a range of behavioural responses that are modulated by and interact with specific neurological and endocrine systems.

Neuroendocrine regulation of social behaviour allows for fine-tuning of the expression of social behaviour in different contexts, which is of particular importance, as the social environment is dynamic and the expression of social behaviour should be adjusted to the prevailing social conditions (Adkins-Regan, 2005; Oliveira, 2009). Hormone levels can be indicators of an individual's internal state, and may regulate the expression of appropriate behaviours under specific physical conditions (Adkins-Regan, 2005; Oliveira, 2009). An example of social behaviour that is regulated by endocrine parameters in order to be adjusted to an individual's internal state and social environment is reproductive behaviour. Reproductive behaviour should take place during fertile stages, while fertile mating partners are present, in order to be effective. During such stages, hormones both signal internal state (fertility) and regulate the expression of appropriate social behaviour under the right social conditions (mating behaviour towards fertile individuals).

Gonadal steroids are important mediators of social interactions, especially in a reproductive context. They are released from the gonads as part of the hypothalamic-pituitary-gonadal (HPG) axis: gonadotrophin-releasing-hormone (GnRH) from the hypothalamus stimulates the pituitary to release luteinizing hormone and follicle stimulating hormone, which in turn stimulate the gonads, leading to the release of

androgens and estrogens (Chaiseha and El Halawani, 2015). There appears to be a general pattern across species, showing that androgens are involved in the regulation of reproduction, competition, aggression and the maintenance of social status in both males and females (see Eisenegger et al. 2011 for review). In birds, competition and density have both been positively correlated to circulating androgen concentrations (Cain and Ketterson, 2012; Ketterson et al., 2005; Langmore et al., 2002; Mazuc et al., 2003; Smith et al., 2005); but see (Cantarero et al., 2015; DeVries et al., 2015; Elekonich and Wingfield, 2000; Jawor et al., 2006b; Schwabl et al., 1988). Furthermore, inter-sexual interactions may affect female androgen levels (e.g. male courtship song (Marshall et al., 2005).

In addition to gonadal steroids, glucocorticoids play an important role in social behaviour (Spencer, 2017). Glucocorticoids are released through activation of the hypothalamic-pituitary-adrenal (HPA) axis, typically in response to challenges and are therefore often called 'stress hormones'. The brain responds to stressors by stimulating the secretion of corticotrophin-releasing from the hypothalamus, which in turn stimulates adrenocorticotrophic hormone (ACTH) secretion from the pituitary. ACTH, in turn, stimulates the release of glucocorticoids from the adrenals. In a social context, social stressors, for example agonistic social interactions, can stimulate the release of glucocorticoids (Creel, 2001; DeVries et al., 2003), yet also buffer the physiological response to stressors (DeVries et al., 2003; Scheiber et al., 2009). In birds, studies have reported a positive correlation between social density and circulating corticosterone concentrations (Cunningham et al., 1987; Koelkebeck and Cain, 1984; Nephew and Romero, 2003; Onbaşılar and Aksoy, 2005; Raouf et al., 2006). In Japanese quail, direct interaction between unfamiliar conspecifics (Rutkowska et al., 2011) as well as unstable social environments (causing an increase in agonistic interactions; (Guibert et al., 2010) increase plasma corticosterone concentrations.

When investigating the effects of environmental influences on an individual's androgen or glucocorticoid levels, it is important to not only take into account baseline levels, but also investigate their respective endocrine axis at different regulatory stages. For example, for glucocorticoids, next to baseline levels, it can be informative to investigate the HPA axis response to an acute stressor (for example, restraint; Astheimer et al., 1995), or to an injection with ACTH, because baseline and maximum response levels can have different effects on behaviour (Creel et al., 2013). Furthermore, the sensitivity of both endocrine axis can be changed after frequent stimulation (HPA: Love et al., 2003; Rich and Romero, 2005; but see Busch et al., 2008; HPG: Peluc et al., 2012) or in response to social stimuli (e.g. for HPA: DeVries et al., 2003; Scheiber et al., 2009; HPG: Lehrman et al., 1961; Stevenson et al., 2008).

Changes in female endocrine parameters conceivably affect reproduction, but the relationships between circulating hormone concentrations and reproduction are highly time- and context-dependent and potentially non-linear, with both very low levels and very high levels negatively affecting reproduction (Bonier et al., 2009a; Hau and Goymann, 2015; Ouyang et al., 2011, 2013). Both androgens and glucocorticoids have important

physiological functions, regulating for example reproductive physiology (Ketterson et al., 2005; Rangel et al., 2006) or metabolism and energy use (Sapolsky et al., 2000). Minimal levels of both circulating androgens and glucocorticoids are therefore required for survival and reproduction, explaining why some studies have reported positive correlations (androgens: Cain and Ketterson, 2012; Langmore et al., 2002; Sandell, 2007; glucocorticoids: Bonier et al., 2009b; Bortolotti et al., 2008; Burtka et al., 2016; Ouyang et al., 2013, 2011. Furthermore, androgens may positively affect reproductive success through their involvement in competitive behaviour, affecting mate and nest acquisition (Cain and Ketterson, 2012; Langmore et al., 2002; Sandell, 2007). However, increased levels circulating androgen or glucocorticoid levels can also negatively affect reproduction (androgens: de Jong et al., 2016; López-Rull and Gil, 2009; Rutkowska et al., 2005; Rutkowska and Cichoń, 2006; Veiga and Polo, 2008; glucocorticoids: Angelier et al., 2010; Bonier et al., 2009b; Ouyang et al., 2013, 2011; Silverin, 1986; Vitousek et al., 2014). These contradictory findings regarding the relation between social stimulation and plasma androgens and glucocorticoids, and their effects on reproduction, indicate that these aspects require further study.

1.2.2. Influences of the social environment on egg composition

Effects of the social environment on female physiology may affect the transmission of resources to the offspring. Understanding how female physiology, the offspring's prenatal environment and subsequently offspring phenotype are related is crucial for studying the proximate mechanisms underlying prenatal maternal effects. In this respect, oviparous species provide an ideal system to study prenatal maternal effects. The fact that the embryo develops outside the mothers body, in a closed environment (the egg) allows for relatively easy assessment and manipulation of the prenatal environment (Henriksen et al., 2011b). In mammals, investigating the offspring's prenatal environment is more complicated due to the relative inaccessibility of the foetal environment, and the variability of the prenatal environment due to maternal and sibling influences.

In oviparous species, the most straightforward assessment of the offspring's prenatal environment is measuring egg size. Determining egg size can give a measure of maternal nutrient provisioning to the offspring, which is an important factor affecting its development. In many avian species, egg size is positively correlated to offspring size or survival (Bernardo, 1996; Krist, 2011; Williams, 1994) and therefore an important mediator of maternal effects.

Effects of the maternal social environment on egg size have been described in a range of avian species. Field experiments in great tits (*Parus major*), for example, have shown a negative correlation between breeding density and egg mass (Perrins and McCleery, 1994). Similar findings have been reported in lab studies in chicken (*Gallus gallus domesticus*) or Japanese quail (*Coturnix japonica*), where keeping females at higher

densities resulted in decreased egg production and egg mass (Anderson et al., 2004; Asghar Saki et al., 2012; Faitarone et al., 2005). However, increased social stimulation may also increase egg mass, as has been shown in lesser black-backed gulls (*Larus fuscus*; Verboven et al., 2005). The direction of effects likely depends on the ecology of the species and type of density measure or social manipulation. Other social factors that may influence female reproductive investment include mate quality (Alonso-Alvarez et al., 2012; Cunningham and Russell, 2000; de Lope and Møller, 1993; Petrie and Williams, 1993; Uller et al., 2005) or female dominance rank (Müller et al., 2002).

Next to nutrients, eggs contain various other compounds from maternal origin, including antioxidants (e.g. carotenoids: Blount et al., 2000; Surai et al., 2001, or vitamins: Surai et al., 1998; Surai and Speake, 1998), immune substances (e.g. maternal antibodies: Buxton, 1952; Hasselquist and Nilsson, 2009, or immunoglobulins: Kowalczyk et al., 1985), and hormones (Schwabl, 1993; von Engelhardt and Groothuis, 2011). Especially maternally derived yolk hormones have been the focus of many studies on maternal effects in avian species. Avian egg yolk contains measurable concentrations of sex steroids from maternal origin, amongst which testosterone, androstenedione, dihydrotestosterone, progesterone and estradiol (Schwabl, 1993; von Engelhardt and Groothuis, 2011). Moreover, studies have detected low levels of corticosterone (Almasi et al., 2012; Rettenbacher et al., 2009). Exposure to maternal hormones during development has important organizational and activational effects on developing tissues, with considerable phenotypic consequences (Groothuis et al., 2005). Furthermore, since maternal circulating hormones fluctuate in response to the maternal environment, hormones are an excellent candidate pathway via which the maternal environment can influence the development and behaviour of offspring (Gil 2003; Groothuis et al. 2005).

Although it is clear that maternally derived yolk hormones are an important mediator of maternal effects, it is still unclear how yolk hormone deposition is regulated. Understanding how hormones accumulate in the egg is important for answering the questions of whether maternal circulating hormones and yolk hormones are independently regulated and whether mothers can control hormone deposition to a certain extent (Groothuis and Schwabl, 2008). Independent regulation of circulating and yolk hormones would enable mothers to vary their yolk hormone content without affecting their plasma hormone levels, the latter potentially affecting behaviour and reproduction, with possible fitness costs (as described in section 1.2.1.). Gonadal steroids in both the maternal circulation and in yolk are produced by the follicular walls of developing oocytes (Groothuis and Schwabl, 2008; Müller et al., 2011). Maternal circulating hormones may therefore simply reflect hormone production in developing oocytes, and correlate with yolk hormone levels. Alternatively, in the case of independent regulation, follicular hormones may be independently distributed to the maternal circulation and to yolk, and circulating and yolk hormone levels are not necessarily correlated. For yolk androgens, positive (Badyaev et al., 2005; Schwabl, 1996a), negative (Mazuc et al., 2003; Navara et al., 2006; Verboven et al., 2003) or no correlations (Goerlich et al., 2010) with maternal circulating levels have been

reported, indicating that independent regulation of circulating and yolk hormones may indeed be possible. Furthermore, when injecting female Japanese quail with radioactively labelled testosterone, only a small amount (0.1%) reached the yolk of their eggs, indeed suggesting that yolk testosterone predominantly originates from the follicular walls surrounding developing oocytes (Hackl et al., 2003).

If yolk testosterone is deposited directly from the follicular walls, stimulating follicular hormone production, for example through activation of the HPG axis via GnRH, should stimulate yolk testosterone deposition. Thus, the magnitude of the androgen response to GnRH may be a better predictor of yolk testosterone concentrations as opposed to maternal baseline plasma testosterone levels (Jawor et al., 2007; Müller et al., 2011; Peluc et al., 2012).

Because the social environment can affect female endocrine physiology, as described in section 1.2.1, it may indirectly affect yolk hormone deposition as well. In a range of avian species, the social environment during breeding has been shown to affect yolk testosterone levels (Bentz et al., 2013; Eising et al., 2008; Hargitai et al., 2009; Mazuc et al., 2003; Pilz and Smith, 2004; Schwabl, 1997; Whittingham and Schwabl, 2002). In house sparrows (*Passer domesticus*), for example, breeding density is positively correlated with yolk testosterone (Mazuc et al., 2003; Schwabl, 1997), and similar findings have been reported for European starlings (*Sturnus vulgaris*; Eising et al., 2008; Pilz and Smith, 2004) and American coots (*Fulica americana*; Reed and Vleck, 2001). In collared flycatchers (*Ficedula abicollis*), although breeding density did not affect yolk testosterone concentrations, when females were exposed to a conspecific same-sex intruder during the nest building period, they laid eggs with higher yolk testosterone levels compared to non-exposed females (Hargitai et al., 2009). Another example of a social factor affecting yolk hormone deposition is the positive relationship between mate quality or attractiveness on yolk testosterone deposition that has been found in a range of avian species (Gil et al., 2004, 1999; Kingma et al., 2009; Loyau et al., 2007).

Social stressors, such as increased levels of agonistic social interactions, may also affect yolk testosterone deposition (Guibert et al., 2010). However, the relationship between maternal stress, plasma corticosterone, and yolk testosterone deposition is still unclear, as studies have reported contrasting results between and even within species. In Japanese quail, for example, repeated mild stressors increased female plasma corticosterone and yolk testosterone concentrations (Guibert et al., 2011), whereas chronic stress reduced yolk testosterone concentrations (Okuliarová et al., 2010). In chicken, elevating female plasma corticosterone via corticosterone implants reduced yolk testosterone levels (Henriksen et al., 2011a). Such contrasting results between studies could be the result of differences in the type and duration/frequency of the applied stress stimulus. Nevertheless, maternal stress has been shown to induce changes in offspring phenotype in many avian species, not only through effects on yolk androgens, but also other measures of egg composition such as egg mass, yolk mass or yolk corticosterone levels (reviewed in Henriksen et al., 2011b).

1.2.3. Evidence for transgenerational effects of the social environment on offspring phenotypes

As described, the social environment of reproducing females can affect their behaviour and physiology, and influence the transmission of resources to the developing offspring. Such changes in the prenatal environment of the offspring can have consequences for their phenotypic development. Transgenerational effects of the social environment have been described in a range of species. As mentioned in section 1.2.2, social stimuli can affect egg composition, with consequences for offspring development. In barn swallows (*Hirundo rustica*), for example, females mated to an attractive male increased their yolk androgen concentrations, which were positively correlated with offspring growth (Gil et al., 2006). Examples showing that the maternal social environment can affect offspring phenotypes have also been reported in other taxa. In American red squirrels (*Tamiasciurus hudsonicus*), for example, exposing mothers to cues signalling a high population density (playback of territorial vocalizations) resulted in faster growing offspring, which may be attributed to effects of increased maternal corticosterone (Dantzer et al., 2013). In guinea pigs (*Cavia aperea*), an unstable maternal social environment affected the offspring in a sex-specific way (Kaiser and Sachser, 2009, 2005). Daughters were more masculinized in their behaviour, and their plasma testosterone concentrations were higher during adolescence. Sons, on the other hand, were infantilized in their behaviour and appeared to have slower sexual maturation, effects that were likely caused by a decrease in maternal plasma androgen concentrations (dehydroepiandrosterone-sulfate and dehydroepiandrosterone) under unstable social conditions (Kaiser et al., 2003; Wewers et al., 2005). In least killifish (*Heterandria formosa*), females that were exposed to a higher social density during brood development produced larger offspring than females experiencing a lower social density (Leips et al., 2009). In desert locusts (*Schistocerca gregaria*), maternal population density affected the behaviour of offspring, with hatchlings from crowded mothers behaving more gregariously, whereas hatchlings from isolated mothers behaved more solitariously (Islam et al., 1994; Simpson et al., 1999). Taken together, these examples indicate an important role of the maternal social environment in offspring phenotypic development.

In some of the mentioned examples, the maternal social environment affected offspring phenotype in a sex-specific way (Kaiser and Sachser, 2009, 2005). Sex-specific maternal effects can be the result of sex-specific allocation of maternal substances, e.g. hormones (Badyaev et al., 2006; Müller et al., 2002) or resources (Young and Badyaev, 2004), but can also arise if the sensitivity to maternal signals differs between male and female offspring (Benowitz-Fredericks and Hodge, 2013; Schweitzer et al., 2013; Tobler and Sandell, 2009; von Engelhardt et al., 2006). Sex-specific maternal investment may lead to sex differences in offspring quality and/or a bias in offspring sex ratio (the proportion of males to females).

Previous studies have shown that the social environment can lead to differences in sex allocation (Michler et al., 2013; Minias et al., 2014) but there is no consistent pattern in the direction of such effects. In great cormorants, for example, social density has been

positively correlated with the proportion of male offspring (*Phalacorcorax carbo sinensis*; Minias et al., 2014). In contrast, in great tits (*Parus major*), increased nesting densities led to female-biased broods in the following year, whereas decreased nesting densities led to male-biased broods (Michler et al., 2013). The specific effect of the maternal social environment on offspring sex ratio likely depends on many different factors, such as the ecology or social organisation of the species.

Sex-specific investment can be adaptive if circumstances are in favour of either one of the sexes. For example, it has been suggested that females in good condition will benefit from investing in the sex with the highest variability in reproductive output in relation to body condition - yielding a high fitness return when in good condition, but a low fitness return when in bad condition (Trivers and Willard, 1973). Biases in offspring sex ratio have been described in a wide range of species, and although the underlying mechanisms remain elusive, many studies have suggested the involvement of maternal condition and circulating hormone levels (Alonso-Alvarez, 2006; Cameron, 2004; Goerlich-Jansson et al., 2013; James, 2008; Krackow, 1995; Navara, 2013; Pike and Petrie, 2003). In avian species, increased levels of maternal androgens usually lead to male-biased offspring sex ratios (Goerlich-Jansson et al., 2013; Goerlich et al., 2009; Pike and Petrie, 2005; Rutkowska and Cichoń, 2006; Veiga et al., 2004, but see Correa et al., 2011). Increased levels of maternal glucocorticoids often result in female-biased offspring sex ratios (Bonier et al., 2007; Goerlich-Jansson et al., 2013; Love et al., 2005; Pike and Petrie, 2006, 2005, but see Gam et al., 2011; Henriksen et al., 2013). However, maternal androgens have also been related to both a female-biased (Correa et al., 2011) as well as an unbiased (Pike and Petrie, 2006) offspring sex ratio, and maternal glucocorticoids have been related to a male-biased (Gam et al., 2011) as well as an unbiased (Henriksen et al., 2013) offspring sex ratio. These contrasting reports indicate that results from experimental and correlational studies are still inconclusive, warranting further research.

1.3. The adaptive significance of maternal effects

Theoretical and experimental studies have proposed different evolutionary implications of maternal effects. Some studies have suggested that maternal effects drive adaptive transgenerational plasticity, enabling parents to prepare offspring for their future environmental conditions and thereby increasing their chances of survival (anticipatory or adaptive maternal effects; Badyaev, 2008; English et al., 2015; Marshall and Uller, 2007; Mousseau and Fox, 1998; Qvarnström and Price, 2001). The example of the transgenerational induction of defences in waterfleas, mentioned at the beginning of this chapter, shows that maternal effects can indeed have adaptive benefits for the offspring (Agrawal et al., 1999). However, maternal effects can also have detrimental consequences for offspring development and survival (Marshall and Uller, 2007). Although maternal effects that negatively affect offspring may seem maladaptive at first, adaptive benefits for mothers may drive these outcomes (Kuijper and Johnstone, 2018; Marshall and Uller, 2007).

In the case of anticipatory maternal effects, their adaptive value is largely dependent on the offspring's environment and how it matches the environmental conditions 'predicted' by the maternal phenotype. If there is a match between the offspring's true environment and the anticipated environment, offspring are predicted to perform better, as they are optimally prepared for the prevailing environmental conditions (Uller et al., 2013). If the actual environment does not match the anticipated environment, offspring are predicted to perform worse (Uller et al., 2013). Maternal and offspring environment are therefore expected to have interactive effects on offspring performance.

Studies of adaptive maternal effects should take these factors into account, and test the outcome of maternal effects under different environmental conditions in the offspring. Ideally, fully-factorial experimental designs should be used, encompassing two maternal and two offspring environments/treatments so the offspring can be tested under matching and mismatching conditions (Engqvist and Reinhold, 2016; Marshall and Uller, 2007; Uller et al., 2013). The advantage of such a match/mismatch setup, which was also used in chapter 4 of this thesis, is that it allows for investigation of interactive as well as independent effects of maternal and offspring environments. A range of fitness-related traits should be quantified, because maternal effects may affect different traits simultaneously, with different results for offspring fitness (Marshall and Uller, 2007; Plaistow and Benton, 2009).

1.4. The Japanese quail as a model for studying maternal effects of the social environment

Japanese quail, *Coturnix japonica* (Figure 1.1), are migratory, ground living birds that belong to the order of Galliformes, in the Phasanidae family. The species has been heavily domesticated, probably already since as early as the eleventh century (Cheng et al., 2010). Originally, Japanese quail were kept for their song, but since the beginning of the twentieth century, quail have been selected for their egg and meat production (Cheng et al., 2010). Though frequently used in behavioural and physiological (or pharmacological) research, studies on the social organization of wild Japanese quail are scarce, and have reported conflicting results. Japanese quail form groups during migration and in winter but may live in pairs during the breeding season (Cheng et al., 2010), and domesticated quail have been shown to form pair bonds (Le Bot et al., 2014). The species has been described as (serially) monogamous, polygynous, and polyandrous and studies have reported a high frequency of extra-pair copulations (Cheng et al., 2010; Mills et al., 1997; Nichols, 1991; Schmid and Wechsler, 1997). Under laboratory conditions Japanese quail are usually housed in polygynous groups. The flexible social organization found in Japanese quail, makes it a suitable species to study the effects of variation in the social environment on physiology and behaviour (Adkins-Regan, 2015; Cheng et al., 2010; Mills et al., 1997).

Japanese quail possess several further traits that make them a very suitable species to investigate transgenerational effects. First, they have a short generation time: the birds reach sexual maturity at about 5-6 weeks of age, and eggs hatch after only 17 days of

incubation. Second, the chicks are precocial and can thus be reared in absence of their mothers, allowing for better standardization of the postnatal environment, avoiding confounding effects of postnatal maternal influences (Henriksen et al., 2011b).

Given these advantages, Japanese quail have been widely used in the study of transgenerational effects (Adkins-Regan et al., 2013; Bertin et al., 2009, 2008; Correa et al., 2011; Guesdon et al., 2011; Guibert et al., 2013, 2012, 2011; Odeh et al., 2003), including maternal effects of the social environment (Guibert et al., 2010). Challenging Japanese quail females with frequent changes in group composition increased their plasma corticosterone concentrations and affected the development and behaviour of the offspring: offspring of mothers kept in unstable social environments developed more slowly during the first weeks of life. Furthermore, chicks from unstable mothers appeared less bold in an emergence test, where the chicks have to leave a box to enter a novel environment, as they had longer latencies to emerge than control chicks. These chicks also reacted more strongly when socially isolated in an unfamiliar environment, as they showed more locomotor acts, started emitting distress calls sooner and made more high posture observations than controls (increased locomotion, distress calls and high posture observations are thought to be signs of active searching for conspecifics, and a high motivation to re-establish social contact; see (Formanek et al., 2008; Guibert et al., 2010). The effects on offspring phenotype appeared to be mediated via changes in yolk testosterone deposition, as females in unstable social environments laid eggs with higher testosterone concentrations in the yolk, when compared to eggs laid by females kept in stable social environments (Guibert et al., 2010).

Studies using Japanese quail selection lines have also suggested a connection between sociality, plasma hormones and yolk hormone deposition. Lines selected for either high or low levels of social reinstatement behaviour (Mills and Faure, 1991) differ in their yolk testosterone deposition. Females from high social reinstatement lines produce eggs containing higher testosterone concentrations than eggs from females from the low social reinstatement line (Gil and Faure, 2007).

Figure 1.1. Adult female (left) and male (right) Japanese quail.

1.5. Thesis aim and outline

The studies described above show that the social environment can affect female physiology, with consequences for reproductive investment and offspring phenotype. However, the social environment entails many different aspects, and their effects are numerous. More research is needed to get a better understanding of how different aspects of the social environment shape maternal physiology and behaviour, and the consequences for fecundity and offspring quality. In addition, the proximate mechanisms underlying maternal effects of the social environment are not yet well understood and require further study.

This thesis focuses on the effects of one aspect of the social environment, namely social group size. We investigated the effects of pair-housing versus group-housing on female physiology, reproductive investment, and subsequently offspring phenotype, also taking into account sex-specific effects and interactions with the offspring's own social environment.

In **chapter 2**, we first studied the effects of pair-housing (one female, one male) versus group-housing (three females, one male) on female physiology and reproductive performance. Increased social stimulation is thought to positively affect circulating and yolk androgen and corticosterone levels, and yolk androgen deposition, which may positively or negatively affect fecundity. We hypothesised that group-housed females would have higher plasma androgen (testosterone and 5-α-dihydrotestosterone) and corticosterone levels, as well as increased yolk testosterone concentrations and investigated whether group- or pair-housing had positive or negative consequences for reproduction. In addition, we tested for effects of the social environment on the female's endocrine responses to standardized challenges. The female's response to a restraint stressor (Wingfield et al., 1995) was measured to test the sensitivity of their HPA-axis. The sensitivity of the female's HPG-axis was also assessed, by measuring their response to an injection with GnRH (Jawor et al., 2006a). Finally, we investigated the idea that the magnitude of the response of the HPG axis to GnRH can be used as a predictor of yolk testosterone deposition (Jawor et al., 2007; Müller et al., 2011; Peluc et al., 2012).

In **chapter 3**, we investigated whether differences in the maternal social environment causes a sex-bias in offspring investment, which can be a result of differential sex allocation, or sex-specific effects on offspring development. Changes in both primary and secondary offspring sex ratio are thought to be mediated by maternal plasma steroids around conception. In avian species, increased levels of maternal androgens are thought to result in male-biased offspring sex ratios whereas increased levels of maternal glucocorticoids have been suggested to result in female-biased offspring sex ratios. We tested the hypothesis that maternal pair-housing or group-housing, and the resulting differences in maternal androgen or corticosterone levels, affects F1 offspring sex ratio, or has sex-specific or overall effects on offspring mortality, growth and circulating androgen or corticosterone levels. In addition, we tested whether the maternal social environment affected the offspring's HPA response to ACTH, which has been suggested to be affected by

maternal social influences (Guibert et al., 2010; Kaiser and Sachser, 2001; von Engelhardt et al., 2015).

In chapter 4 we studied the effects of the maternal social environment on the adult female offspring, housed under different social conditions: in pairs of two females or in groups of four females (Figure 1.2). The adaptive value of maternal effects is thought to depend on how well the offspring's environment matches the environmental conditions 'predicted' by the maternal phenotype. Our setup allowed us to test whether offspring kept in an environment that matched the maternal environment with respect to social group size (two versus four individuals) perform better than offspring kept under mismatching social conditions, that is, grow more and have a higher reproductive success. On the other hand, we were able to investigate whether the maternal social environment affects offspring phenotype independently of the offspring's social environment, and vice versa. As in chapter 1, we also measured the female's circulating androgen and corticosterone levels and tested the sensitivity of their HPA and HPG axis.

In **chapter 5**, the findings of this thesis are discussed, while providing ideas for follow-up experiments to further unravel the mechanisms behind, and consequences of, maternal effects of the social environment.

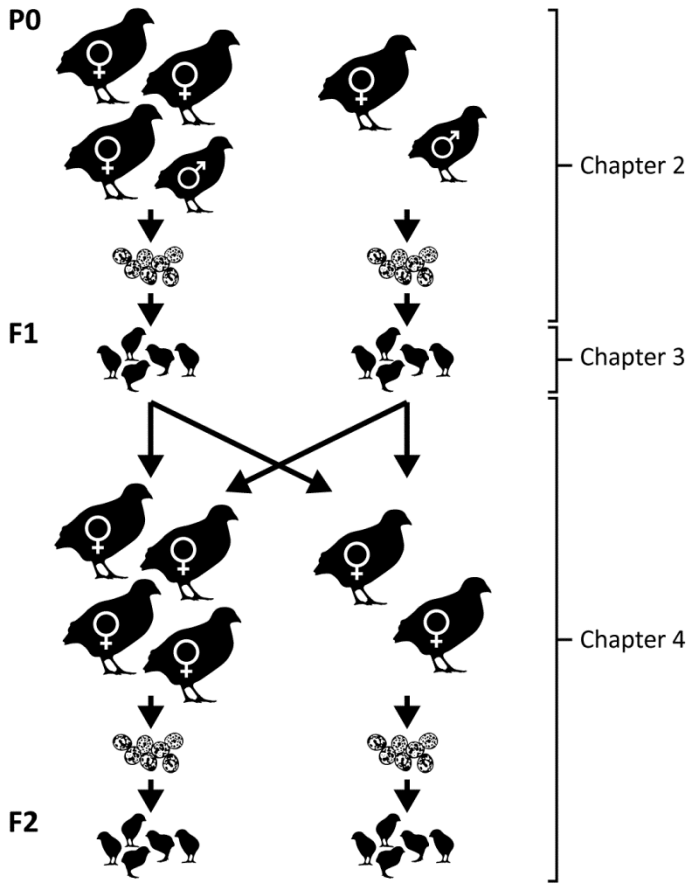

Figure 1.2. Graphical representation of the used experimental setup. See text for further details.

Chapter 2

Social environment during egg laying: changes in plasma hormones with no consequences for yolk hormones or fecundity in female Japanese quail, *Coturnix japonica*

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Abstract

The social environment can have profound effects on an individual's physiology and behaviour and on the transfer of resources to the next generation, with potential consequences for fecundity and reproduction. However, few studies investigate all of these aspects at once. The present study housed female Japanese quail (*Coturnix japonica*) in pairs or groups to examine the effects on hormone concentrations in plasma and yolk and on reproductive performance. Circulating levels of androgens (testosterone and 5-α-dihydrotestosterone) and corticosterone were measured in baseline samples and after standardised challenges to assess the responsiveness of the females' endocrine axes. Effects of the social environment on female fecundity were analysed by measuring egg production, egg mass, fertilization rates, and number of hatched offspring. Counter to expectation, females housed in pairs had higher plasma androgen concentrations and slightly higher corticosterone concentrations than females housed in groups, although the latter was not statistically significant. Pair vs. group housing did not affect the females' hormonal response to standardised challenges or yolk testosterone levels. In contrast to previous studies, the females' androgen response to a gonadotropin-releasing hormone challenge was not related to yolk testosterone levels. Non-significant trends emerged for pair-housed females to have higher egg-laying rates and higher fertility, but no differences arose in egg weight or in the number, weight or size of hatchlings. We propose that our unexpected findings are due to differences in the adult sex ratio in our social treatments. In pairs, the male may stimulate female circulating hormone levels more strongly than in groups where effects are diluted due to the presence of several females. Future studies should vary both group size and sex composition to disentangle the significance of sexual, competitive and affiliative social interactions for circulating and yolk hormone levels, and their consequences for subsequent generations.

2.1. Introduction

The social environment of an individual can profoundly affect its behaviour, morphology and physiology. In many vertebrates, including birds, the frequency and type of social interactions affect circulating androgen and glucocorticoid levels (Adkins-Regan, 2005; Creel et al., 2013; Oliveira, 2004). Social interactions influence steroid hormones which, in turn, affect social and reproductive behaviour (Lehrman, 1964; Lehrman et al., 1961). Hence steroid hormones can act as mediators between the social environment and behaviour (Adkins-Regan, 2005; Oliveira, 2009), which ultimately can affect survival and reproduction (Dufty et al., 2002; Ketterson and Nolan Jr., 1992). During reproduction, the social environment not only affects the individual itself, but also the amount of resources and other substances transferred to the next generation, potentially affecting offspring fitness (Groothuis et al., 2005; Kaiser and Sachser, 2005). Such socially induced maternal effects enable parents to prepare offspring for their future social conditions, potentially resulting in adaptive transgenerational plasticity (Badyaev, 2008; English et al., 2015; Mousseau and Fox, 1998; Qvarnström and Price, 2001; but see Marshall and Uller, 2007). The mechanisms underlying the effects of the social environment on female physiology and behaviour and the consequences for fecundity and offspring quality are not yet well understood and deserve further research.

Gonadal steroids, regulated by the hypothalamic-pituitary-gonadal (HPG) axis, are important mediators of social interactions, especially in a reproductive context. Androgens, in particular testosterone (T), are involved in social interactions such as competition and aggression, as well as reproductive behaviour and physiology (Adkins-Regan, 2005; Oliveira, 2004). In birds, female plasma androgen levels have been found to be positively correlated with conspecific competition and breeding density (Cantarero et al., 2015; Langmore et al., 2002; Mazuc et al., 2003; Smith et al., 2005; Zysling et al., 2006). The link between plasma androgen levels and intra-sexual competition has been extensively studied under the "challenge hypothesis" (Wingfield et al., 1990), which states that, during reproduction, plasma T correlates positively with male-male competition. In females, although there are fewer studies than in males, similar hormonal responses to social challenges have been observed (Cain and Ketterson, 2012; Ketterson et al., 2005; Langmore et al., 2002; Smith et al., 2005), yet studies have also reported no link, or even negative correlations between female-female competition and circulating plasma androgen levels (Cantarero et al., 2015; DeVries et al., 2015; Elekonich and Wingfield, 2000; Jawor et al., 2006b; Schwabl et al., 1988). Given these contradictory findings, further research is required to clarify the relationship between intra-sexual competition and circulating androgens in females.

Next to gonadal steroids, glucocorticoids play an important role in social behaviour. In avian species, corticosterone (CORT) is typically released under metabolic or otherwise challenging conditions, through activation of the hypothalamic-pituitary-adrenal (HPA) axis, and is therefore often referred to as a 'stress hormone'. Socially challenging interactions can stimulate the HPA axis and increase circulating glucocorticoid levels, while affiliative social interactions can buffer the response to stressors (Creel et al., 2013; Hennessy et al., 2009). In birds, social density and circulating baseline CORT concentrations frequently are

positively correlated (Cunningham et al., 1987; Koelkebeck and Cain, 1984; Onbaşılar and Aksoy, 2005; Raouf et al., 2006; but see Davami et al., 1987; Koelkebeck and Cain, 1984; Poot et al., 2012). In Japanese quail (*Coturnix japonica*), females housed in unstable social environments have higher plasma CORT concentrations following changes to the social environment compared to females kept in stable social environments (Guibert et al., 2010). Moreover, social interactions between Japanese quail females and an unfamiliar conspecific result in elevated CORT levels (Rutkowska et al., 2011).

Circulating levels of androgens and CORT can affect female behaviour and reproductive investment, thereby influencing reproductive success both positively and negatively. Artificially elevated female plasma androgens have been shown to negatively affect reproduction (de Jong et al., 2016; López-Rull and Gil, 2009; Rutkowska et al., 2005; Rutkowska and Cichoń, 2006; Veiga and Polo, 2008), although the long-term effects on lifetime reproductive success may be small (Veiga and Polo, 2008). However, circulating androgens may have indirect positive effects on female reproductive success, for example by affecting competition, mate and nest acquisition and parental behaviour (Cain and Ketterson, 2013, 2012; Langmore et al., 2002; Sandell, 2007; Searcy, 1988). Circulating female CORT levels have been found to both negatively (Angelier et al., 2010; Bonier et al., 2009b; Ouyang et al., 2013, 2011; Silverin, 1986; Vitousek et al., 2014) and positively correlate with reproduction (Bonier et al., 2009b; Burtka et al., 2016; Ouyang et al., 2013, 2011). In Japanese quail, selection lines bred for an exaggerated stress response showed a decrease in reproductive success, with an additional negative effect of artificially increasing CORT levels in these females (Schmidt et al., 2009). It is still unclear what causes the variable effects of androgens and CORT on reproduction. Possible explanations include context-dependent effects, time-dependent effects and non-linear effects of increasing hormone concentrations (Bonier et al., 2009a; Hau and Goymann, 2015; Ouyang et al., 2013, 2011).

In reproducing female birds, not only are plasma levels of steroids affected by the social environment, but also the deposition of hormones into yolk of developing eggs (Gil, 2008; Groothuis et al., 2005; von Engelhardt and Groothuis, 2011). Breeding density and female-female competition is positively correlated with yolk androgens in many bird species (Bentz et al., 2013; Eising et al., 2008; Hargitai et al., 2009; Mazuc et al., 2003; Pilz and Smith, 2004; Schwabl, 1997; Whittingham and Schwabl, 2002). In the Japanese quail, yolk androgens are increased by social instability (Guibert et al., 2010) and by selection for a high motivation to reinstate social contact (Gil and Faure, 2007). The relationship between female plasma androgen levels and yolk androgen levels is still unclear (Groothuis and Schwabl, 2008; Moore and Johnston, 2008), but recent studies have suggested that variation in yolk hormone levels reflects differences in HPG axis sensitivity. Indeed, the increase of circulating androgens in response to gonadotropin-releasing hormone injections (GnRH) correlates positively with yolk androgen deposition in some bird species (Jawor et al., 2007; Müller et al., 2011), including Japanese quail (Peluc et al., 2012). This suggests a link between the social environment, the plasma androgen response to GnRH and yolk androgen levels.

Steroid hormones in the yolk influence the development and behaviour of offspring and are therefore important mediators of prenatal maternal effects. Yolk androgens influence fundamental traits such as offspring growth (both pre-and post-natal), timing of hatching, offspring immunity and behaviour (Gil, 2003; Groothuis et al., 2005). These factors can have consequences for offspring survival thus ultimately affecting the parents' reproductive success (Gil, 2003; Groothuis et al., 2005).

Given the contradictory findings on the relation between social stimulation, plasma androgens, CORT and yolk androgens, and their effects on reproduction, we explored the effects of the social environment in captive housed female Japanese quail. We kept the birds either in pairs (one male and one female) or in small groups of three females with one male to represent variation in the social system during breeding which may be found in the wild and in captivity. Japanese quail have been described as (serially) monogamous, polygynous, and polyandrous (Cheng et al., 2010; Mills et al., 1997; Nichols, 1991). Studies on domesticated Japanese quail have shown that formation of (temporary) pair bonds indeed occurs, but the frequency of extra-pair copulations is high, and under laboratory conditions this species is usually housed in polygynous groups (Cheng et al., 2010; Mills et al., 1997; Nichols, 1991; Schmid and Wechsler, 1997). Overall, this suggests that the mating system is flexible in Japanese quail, which makes this a suitable species to study the effects of variation in the social environment on physiology and behaviour (Adkins-Regan, 2015; Cheng et al., 2010; Mills et al., 1997).

Compared to pair housing, group living should allow for more social interaction and potentially increase competition for resources and the male mating partner. These social stimulations are expected to result in changes in plasma and yolk hormone levels. We analysed the effect of social housing conditions on female plasma androgen and corticosterone levels and yolk T concentrations in their eggs. We refer to plasma androgens rather than T because the assay used cross-reacted to 23.3% with 5-α-dihydrotestosterone (5-α-DHT; see Methods), a potent androgen present in avian plasma (Balthazart et al., 1983; Feder et al., 1977; Roy et al., 1998). To investigate the effects of the social environment on HPG and HPA axis sensitivity, we tested the females' physiological response to specific challenges. We subjected females to a standardized restraint stress protocol (Wingfield et al., 1995), allowing us to measure the CORT response to a stressor via activation of the HPA axis. We also performed a GnRH challenge, testing the sensitivity of the HPG axis (Jawor et al., 2006a). Finally, we analysed the effects of the social environment on female fecundity by measuring egg production and egg mass, fertilization rate, and number of offspring hatching in the F1 generation. To analyse potential differences between females within groups due to variation in affiliative and sexual interactions, we recorded social proximity and female baldness caused by repeated copulation with the male (Kovach, 1974; Mills et al., 1997).

We predicted group housing would result in elevated plasma androgen and CORT levels and higher yolk T levels due to the increased amount of social stimulation. We expected the change in circulating hormone levels to affect reproductive performance,

however, given the variable results reported in the literature, we did not have a clear prediction regarding the direction of effects.

2.2. Methods

2.2.1. Experimental design

The experiment was conducted using a total of 96 animals. At 29 days of age, when the birds had developed their sexually dimorphic plumage but were not yet sexually mature, they were placed in the social treatment conditions. Groups consisted of three females and one male, while pairs included one female and one male. The birds were allocated as follows: 36 females and 12 males to 12 groups, 24 females and 24 males to 24 pairs. Siblings and half-siblings were equally distributed over the two social treatments and never housed in the same cage, in order to balance out potential genetic effects on endocrinology and reproduction. The distribution of the cages within the two experimental rooms was balanced for treatment. Measures of the females' physiology, behaviour and reproduction were taken at different time points as described below (for an overview see Figure 2.1). Animals were weighed at the start of treatment (day 29), after five weeks into the treatment (day 65) and at the end of the experiment (day 87).

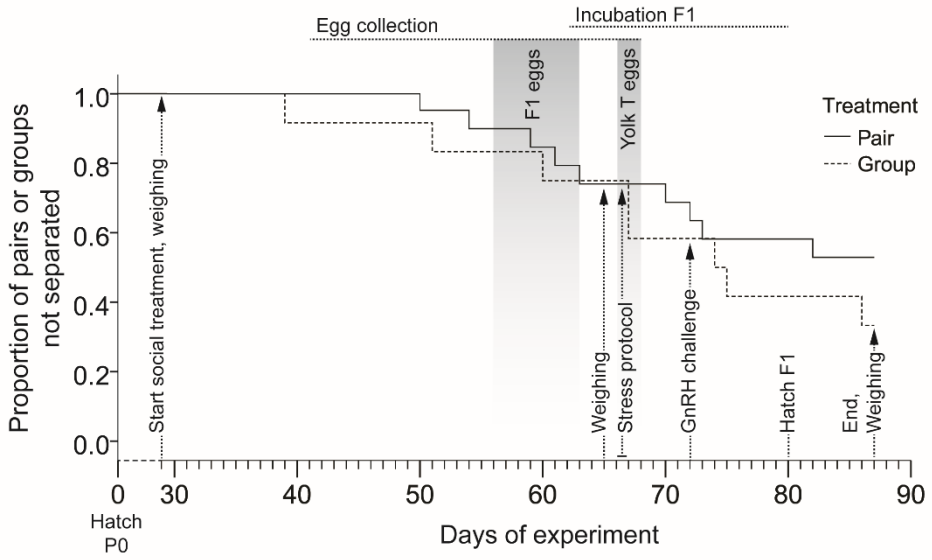

Figure 2.1. Timeline of experimental procedures, and separations of pairs and groups.

Due to aggression, we had to separate three pairs using a wire mesh which still allowed acoustic, visual, olfactory and limited tactile interaction. Two groups also had to temporarily be wire separated due to aggression (one animal on one side of the wire, the other three animals on the other side). Wire separated animals were included in our analyses and excluding these animals from our statistical analyses did not qualitatively change the results. Eight groups – including the two groups that had already been wire separated - and eleven pairs had to be completely removed from the experiment between

day 30 and day 87 because at least one bird in the cage had wounds that were unlikely to heal within a few days, constituting a humane endpoint. In addition, in two pairs the male died, hence the females were excluded from all analysis of parameters following the death. As a consequence, sample size varies for different measures (table 1). However, the reduction of sample size over the course of the experiment did not differ between social treatments (Kaplan-Meier survival analysis using Breslow test statistics: $\chi^2_1 = 1.06$, $p = 0.30$; Figure 2.1).

Table 2.1. Measures of pair-housed and group-housed females and their offspring, with sample sizes.

Measure		Pair-housed females				Group-housed females			
		Mean ± 1 SEM	n	Excluded	Missing sample	Mean ± 1 SEM	n	Excluded	Missing sample
Female weight, day 65		235.09 g ± 5.35	17 ♀♀	7 ♀♀		230.10 g ± 2.60	33 ♀♀ 11 groups	3 ♀♀ 1 group	
Female weight, day 87		243.85 g ± 6.64	13 ♀♀	11 ♀♀		241.67 g ± 6.41	12 ♀♀ 4 groups	24 ♀♀ 8 groups	
Age at first egg		45.48 days ± 0.74	23 ♀♀	1 ♀		45.47 days ± 0.57	36 ♀♀ 12 groups		
Total nr of eggs collected		0.66 eggs/♀/day ± 0.02	361 eggs 23 ♀♀	1 ♀		0.60 eggs/♀/day ± 0.02	533 eggs 12 groups		
Egg mass		10.17 g ± 0.06	324 eggs 23 ♀♀	1 ♀		10.33 g ± 0.04	531 eggs 12 groups		
Stress protocol	Baseline	2.77 ng/ml ± 0.22	14 ♀♀	6 ♀♀	4 ♀♀	2.12 ng/ml ± 0.14	24 ♀♀ 11 groups	3 ♀♀	9 ♀♀
	Post-challenge	13.05 ng/ml ± 1.66	14 ♀♀			11.05 ng/ml ± 1.65	22 ♀♀ 11 groups	1 group	11 ♀♀
Yolk mass		2.78 g ± 0.04	51 eggs 18 ♀♀	6 ♀♀		2.78 g ± 0.03	73 eggs 10 groups	6 ♀♀ 2 groups	
Yolk T	Concentration	5.02 pg/mg ± 0.89	17 eggs	6 ♀♀	1 ♀	5.81 pg/mg ± 0.86	24 eggs	6 ♀♀	
	Total	14.07 ng ± 2.55	17 ♀♀			15.86 ng ± 2.29	10 groups	2 groups	
GnRH challenge	Baseline	0.67 ng/ml ± 0.05	16 ♀♀	7 ♀♀	1 ♀	0.51 ng/ml ± 0.03	22 ♀♀ 8 groups	12 ♀♀	2 ♀♀
	Post-challenge	0.72 ng/ml ± 0.05	16 ♀♀			0.56 ng/ml ± 0.03	22 ♀♀ 8 groups	4 groups	
Eggs for F1	Eggs collected	6.89 eggs/♀ ± 0.31	124 eggs	5 ♀♀	1 ♀[1]	6.46 eggs/♀ ± 0.44	155 eggs	6 ♀♀	6 ♀♀[1]
	Eggs fertilized	6.06 eggs/♀ ± 0.30				5.08 eggs/♀ ± 0.55			
	Eggs hatched	3.72 eggs/♀ ± 0.46	18 ♀♀			2.88 eggs/♀ ± 0.52	8 groups	2 groups	
F1 offspring	Mass at hatching	7.18 g ± 0.08	66 chicks	5 ♀♀	2 ♀♀[1, 2]	7.10 g ± 0.06	69 chicks	6 ♀♀	10 ♀♀[1, 2]
	Tarsus at hatching	12.93 mm ± 0.06	17 ♀♀			12.77 mm ± 0.07	20 ♀♀ 8 groups	2 groups	

[1]male infertile; [2]females without offspring

2.2.2. Animal husbandry

The Japanese quail originated from eggs generously provided by the INRA in Nouzilly, France (Experimental unit 1295 (UE PEAT) and UMR 85, Physiologie de la Reproduction et des Comportements, INRA-CNRS-IFCE-Université de Tours, Val de Loire Center, Nouzilly, France). Eggs were laid by females from a non-selected control line, bred next to quail lines selected for low or high social reinstatement (Mills and Faure, 1991). At the INRA, each cage housed two females and one male, thus housing conditions were intermediate compared to the conditions used in this study. All eggs were incubated at the same time, hatched, and birds reared at Bielefeld University, Germany.

The experiments were performed in two adjacent indoor rooms with artificial lighting and no natural daylight. The light-dark cycle was 14:10 h (lights on at 5:00 am, lights off at 7:00 pm), and the rooms had ambient temperature with additional heating to maintain at least 20˚C. Cages for pairs measured 75 x 80 x 40 cm, group cages 150 x 80 x 40 cm. None of the cages faced each other to prevent visual contact between birds from different cages, but acoustic and olfactory communication was possible. The birds were kept on wood shavings, and all cages contained a sand bath and one shelter hut per female. Feed (GoldDott Hennenmehl, Derby Spezialfutter GmbH, Münster, Germany) and water was provided ad lib. The standard diet was supplemented on a weekly basis with mealworms and shell grit.

2.2.3. Ethics statement

All experimental procedures and humane endpoints for minimizing suffering were approved by the North Rhine-Westphalia State Agency for Nature, Environment and Consumer Protection (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen), Recklinghausen, Germany (licence number 84-02.04.2013-A127). Animal facilities were approved for keeping and breeding Japanese quail for research purposes by the local government authority responsible for health, veterinary and food monitoring (Gesundheits-, Veterinär- und Lebensmittelüberwachungsamt Bielefeld, Germany).

2.2.4. Egg collection

All cages were checked for eggs daily from day 39 after hatching, 10 days after birds had been placed in the experimental groups and before any egg had been laid. Eggs were collected until day 68 and all eggs were weighed to the nearest 0.01 g. Eggs collected until day 56 were used to analyse the onset of egg laying in pair-housed vs. group-housed females. Since we could not identify which female laid an egg in a group, we recorded the day at which we found the first, second and third egg in a group as the age at which the first, second and third female had started laying eggs. Eggs collected between day 56 and day 63 were artificially incubated to produce the F1 generation (see table 2.1 for sample sizes). Eggs from days 66-68 were frozen at -20 directly after collection and later used to determine yolk T concentrations (see table 2.1 for sample sizes). Most eggs for yolk T measurements were collected in the morning before the stress protocol (n = 31), or the

morning of the day after the stress protocol (n = 7). This means that at the time of the stress protocol, most eggs for yolk T measurements had already been laid or ovulated, and yolk T levels were unlikely to be affected by the stress protocol (in quail, ovulation usually takes place in the afternoon, approximately 15-30 minutes after oviposition of the previous egg (Bacon and Koontz, 1971; Houdelier et al., 2002; Woodard and Mather, 1964). Only one egg was collected in the morning two days after the stress protocol, but here too the yolk should have been almost completely formed at the time of the stress protocol. Since deposition of yolk T is repeatable within individual females (Okuliarová et al., 2009), we expected the yolk T measurements to be representative for all eggs that an individual female laid during the course of this study. All females continued laying until the end of the experiment (day 87). In total, we collected 361 eggs from pair females and 533 eggs from group females (see table 2.1).

2.2.5. Egg incubation and hatching

Eggs were incubated in complete darkness in a HEKA-Euro-Lux II incubator (HEKA-Brutgeräte, Rietberg, Germany). Until incubation day 14, the temperature was set at 37.8°C, humidity to 55% and eggs were turned for 30 minutes every 2 hours. Candling of the eggs was done after 9 days of incubation, and infertile eggs were removed. On day 15 of incubation, eggs were moved to hatching trays, the incubation temperature was set to 37.5°C, the humidity to 75%, and eggs were no longer turned. On average, chicks hatched after 17 days of incubation (range: 16-18 days). Chicks were removed from the incubator once their feathers had dried (ca. 2 hours after hatching). Upon removal from the incubator, all chicks were weighed to the nearest 0.1 g and tarsus was measured to the nearest mm using a digital calliper. Parentage for offspring of group-housed mothers was ascertained by genotyping all parents and chicks. A small blood sample (max. 50 µl) was taken from the chicks on the day of hatching by pricking the jugular vein with a 27 gauge needle and collecting the blood in heparinized capillaries (BRAND GMBH + CO KG, Wertheim, Germany). From the parental birds, a small sample of blood from the stress protocol or GnRH challenge were used. Blood was diluted 1:2 with phosphate buffer saline (10 mM PBS+6 mM EDTA, pH 7.4) and stored at -20°Celsius.

For genotyping, genomic DNA was obtained by a phenol/chloroform or Chelex extraction (Walsh 1991). We genotyped all individuals at 22 microsatellite loci using fluorescently labelled primers selected from a previous study (Kayang et al., 2002) in three separate multiplexed PCR reactions: mix 1 (GUJ0001, GUJ0011, GUJ0028, GUJ0044, GUJ0068, GUJ0085, GUJ0097, GUJ0100; annealing temperature 55° Celsius), mix 2 (GUJ0021, GUJ0029, GUJ0062, GUJ0065, GUJ0069, GUJ0074, GUJ0083, GUJ0094; annealing temperature 60° Celsius), mix 3 (GUJ006, GUJ0054, GUJ0057, GUJ0071, GUJ0077, GUJ0092; annealing temperature 55 °Celsius). DNA was amplified using the Type-It Kit (Qiagen) in 10 µl reactions (1 µl DNA, 1 µl primer-mix, 3.5 µl Type-It mastermix, 4.5 µl water), following the manufacturer's PCR protocol (one cycle of 5 min at 94°C; 28 cycles of 30 s at 94°C, 90 s at the annealing temperature and 30 s at 72°C; and one final cycle of 15 min at 72°C). PCR products were separated by electrophoresis on a capillary sequencer (ABI 3730xl), fragment

sizes were scored automatically using GeneMarker v1.95 and checked manually for errors. Paternity was manually assigned by identifying which genotype of the three potential mothers in a cage matched the offspring genotype.

2.2.6. Stress protocol

Female CORT baseline and response values after a stressor were assessed in a standardised restraint stress protocol on days 66-67. All birds were tested between 09:15 am - 12:30 pm and CORT levels did not change significantly during that period ($F_{(1, 30.89) \text{ time of day}} = 2.83$, $p = 0.10$). Birds were caught, and a blood sample was taken within 3 minutes to determine baseline plasma CORT concentrations. Blood sampling was done by puncturing the ulnar vein with a sterile needle and collecting 200-300 µl blood in heparinised capillaries (BRAND GMBH + CO KG, Wertheim, Germany). Following the baseline sample, the birds were restrained for 10 minutes by placing them in bird holding bags (Ecotone, 25x30 cm), after which a second blood sample was taken to determine the CORT response to restraint.

2.2.7. GnRH challenge

On day 72 we measured the females' baseline plasma androgen concentrations, and their response values following a GnRH injection while females were still laying eggs. To exclude effects of the GnRH injection itself on yolk hormone levels or reproduction, the GnRH challenge was performed after collecting the eggs for the next generation and for yolk T measurements (Peluc et al., 2012). All birds were tested between 10:00 am - 15:30 pm. As in the stress protocol, animals were caught, and a blood sample was taken from the ulnar vein within 3 minutes to determine baseline plasma androgen concentrations. After the baseline sample was taken, the females were injected in the pectoral muscle with 5 µg chicken GnRH-I (H-3106, APC number 54-8-23, *CAS No: 47922-48-5,* Bachem, Bubendorf, Switzerland, formerly also sold as Sigma-L0637) dissolved in 50 µl PBS, based on a protocol previously used in quail (Peluc et al., 2012), and returned to their home cages. Thirty minutes post injection, the birds were caught again, and a second blood sample was taken to determine the androgen response to GnRH.

2.2.8. Social proximity and baldness scores

To assess social proximity, all cages were checked once a day in the morning from day 45 to day 63 (except for weekends, resulting in 16 daily checks) to note which individuals were sitting together (within the space of one quail body length from each other). We then calculated how often a female sat with the male and, in groups, how often a female sat with at least one other female.

As a measure of male copulatory behaviour with the female, we classified females as 'bald' or 'not bald' depending on whether feathers were missing from the back of their head or their back at the end of the experiment (on day 87 or on the day of separation for separated animals). Male Japanese quail grab the female's head or neck feathers during copulation and then mount her back (Kovach, 1974; Mills et al., 1997). Hence, baldness

occurs following frequent copulations. We did not score baldness on a location other than the back of the head or the back involving skin damage since this is most likely caused by aggressive pecking.

2.2.9. Plasma corticosterone and androgens

Blood samples from both the stress protocol and the GnRH challenge were kept on ice for a maximum of two hours after sampling and then centrifuged for 10 minutes at 2000 x g. Following centrifugation, plasma was collected and frozen at -20°C.

Plasma CORT concentrations were determined using a commercial corticosterone radioimmunoassay kit (MP Biomedicals, Orangeburg, USA, cat. no. 07-102102). Cross-reactivity of the kit antibody, as reported by the manufacturer, was 0.34% for desoxycorticosterone, 0.1% for testosterone, and less than 0.1% for other steroids. Samples were balanced for treatment across assays. Samples were measured together with quail plasma samples from other experiments and all were distributed over 10 assays with an average intra-assay coefficient of variation (CV) of 4.78%, and an inter-assay CV of 7.13% (based on a chicken plasma pool and 2 kit controls measured in each assay).

Plasma androgen concentrations were determined using a commercial T enzyme immunoassay kit (Demeditec Diagnostics GmbH, Kiel, Germany, cat. no. DES6622). Cross-reactivity of the kit antibody, as reported by the manufacturer, was 23.3% for 5α-dihydrotestosterone, 1.6% for androstenedione, and less than 0.1% for other steroids. Control plasma pool samples were incorporated in each run. Samples from the third assay were significantly higher than samples measured in the first two assays (effect of assay: $F_{(2, 18.01)} = 5.93$, $p = 0.01$). Since eight samples (four each from assay one and two) were re-measured in assay three, we could correct the values from the third assay using a regression of the measures for samples re-measured in assay three on their corresponding values from assays one and two. Excluding samples from assay three did not change the results qualitatively. After correction, the average intra-assay CV was 5.84% and the inter-assay CV was 9.77%.

2.2.10. Yolk testosterone

Yolk preparation and extraction was based on previously established methods (Goerlich et al., 2012). In preparation for extraction, the frozen yolk was separated from the albumen and egg shell and weighed to the nearest 0.01 g. Yolks were homogenized with 4 ml distilled water and then stored at -20°C.

For T extraction, 100 mg of the yolk-water mix was transferred to a 2 ml Eppendorf tube and further diluted with 100 µl distilled water. All samples were then spiked with 4000 cpm of tritium-labelled T (Perkin Elmer NET553250UC), vortexed and incubated for 30 minutes in a 37˚C water bath. After incubation, 500 µl 100% ethanol was added, and samples were vortexed for 15 minutes. After vortexing, samples were centrifuged for 10 minutes at 4˚C and 15800 x g. The supernatant was then decanted into a fresh 2 ml Eppendorf tube and frozen overnight on dry ice. The next day, samples were centrifuged

again for 10 minutes at -9˚C and 15800 x g, and the supernatant was decanted into fresh tubes. Samples were then dried in a vacuum concentrator (approximately 2 hours) and the pellet was re-dissolved in 500 µl steroid-free human serum (IBL international, RE52999). For determination of extraction efficiency, 30 µl of each sample was counted in a beta counter. Recoveries were on average 82.5 ± 0.8% (mean ± 1 SEM).

A commercial radioimmunoassay kit was used to determine yolk T concentrations (DIAsource ImmunoAssays S.A., Louvain-la-Neuve, Belgium, cat. no. KIP1709). Cross-reactivity of the kit antibody, as reported by the manufacturer, was 0.31% for dihydrotestosterone, 0.28% for androstenedione and less than 0.1% for other steroids. All samples were measured in a single assay, the intra-assay CV was 3.86%. Total yolk T was calculated by multiplying the yolk T concentrations with total yolk mass.

2.2.11. Statistical analyses

Statistical analyses were performed using R 3.2.3 (R Development Core Team 2015). General linear mixed models were fitted for plasma hormones, yolk T, growth, onset of egg laying, egg mass, yolk mass and F1 mass and tarsus at hatching (calculated using lmer from the lme4 package in R, using the package lmertest to extract F values and p values). Pearson's correlation coefficient was used to analyse the correlation between baseline and post-challenge androgens and CORT. Generalised linear mixed models (calculated using glmer from R's lme4 package) with a binomial distribution and logit link function were calculated for analysis of egg laying, fertilization and hatching success.

For the analysis of hormonal responses to the GnRH challenge and the stress protocol, we used cage and individual identity (ID) nested within cage as random effects and social treatment (treatment) and sample number (sample) as fixed predictors, as well as the treatment by sample interaction (treatment * sample).

To analyse potential effects of copulation with the male, or social proximity on female baseline plasma hormone concentrations, we fitted models on baseline androgen and CORT concentrations, with female baldness (baldness; since there were only three non-bald pair-housed females, they were excluded from the analysis and we compared three categories: bald pair-housed females, and bald and non-bald group-housed females) or social proximity (sitting with male or sitting with female) as fixed predictors. Least-significant-difference post-hoc tests were used to test which categories differed from each other. All models included cage as a random effect.

Yolk T was analysed using cage as a random effect and treatment as a fixed effect, and to test the relationship between plasma androgens and yolk T, the models included either average baseline androgen concentration (baseline androgens) or average plasma androgen increase in response to GnRH (Δ androgens in response to GnRH) from each cage as an additional predictor. The average female plasma androgen concentration per cage was used since we were unable to assign eggs to individual females in groups.

Analyses of female mass included cage and individual ID nested within cage as random effects, treatment and female age (age) as fixed predictors, and female mass at the start of the social treatment (day 29) as a covariate.

Models analysing the onset of egg laying (age at first egg) included a random effect of cage, and social treatment as a fixed predictor.

Models for egg laying rate and egg mass included a random effect of cage and a fixed effect of social treatment. In addition, the models analysing egg laying rates included a linear, quadratic and cubic effect of collection day (collection day + collection day^2 + collection day^3) to model the non-linear relationship between age and egg laying rates. Likewise, the models for the analysis of egg mass included a linear, quadratic and cubic effect of the days since the onset of laying for each cage (day+day^2+day^3). Moreover, egg laying rate models included a treatment by age interaction effect (treatment *(collection day + collection day^2 + collection day^3)), and egg mass models an effect of the interaction of treatment and days since the onset of egg laying (treatment * (day+day ^{2}+day^3)).

Yolk mass was only measured in the subset of eggs collected for yolk hormone measurements, and models for the analysis of yolk mass included a random effect of cage and a fixed effect of social treatment.

Fertilization and hatching success were analysed using cage as a random effect and treatment as a fixed effect.

F1 mass and tarsus at hatching included maternal cage and maternal ID nested within maternal cage as random effects (we were able to include maternal ID since we had assigned chick parentage) and treatment as a fixed effect.

We started out with the full models including all interactions and then excluded stepwise all non-significant predictors/interactions ($p > 0.05$), except for the main terms of interest, i.e. social treatment and sample number (for hormonal responses). Distributions of model residuals were tested using Kolmogorov-Smirnoff tests and visually assessed using histograms and Q-Q plots. Plasma CORT concentrations were log-transformed, and yolk T concentrations were square-root transformed to achieve normality. No transformation was used when analysing the correlation between baseline plasma androgens and CORT.

2.3. Results

2.3.1. Androgens

Overall, pair-housed females had significantly higher plasma androgen concentrations than group-housed females ($F_{(1,\ 11.40)\ Treatment} = 7.88$, $p = 0.02$; table 2.1, Figure 2.2A). GnRH injections resulted in a small, but significant, increase in plasma androgen concentrations ($F_{(1,\ 37)\ Sample} = 6.46$, $p = 0.02$; table 2.1, Figure 2.2A), but the response to GnRH did not differ between pair-housed and group-housed females ($F_{(1,\ 36)\ Treatment\ *\ sample} < 0.01$, $p = 0.98$; Figure 2.2A).

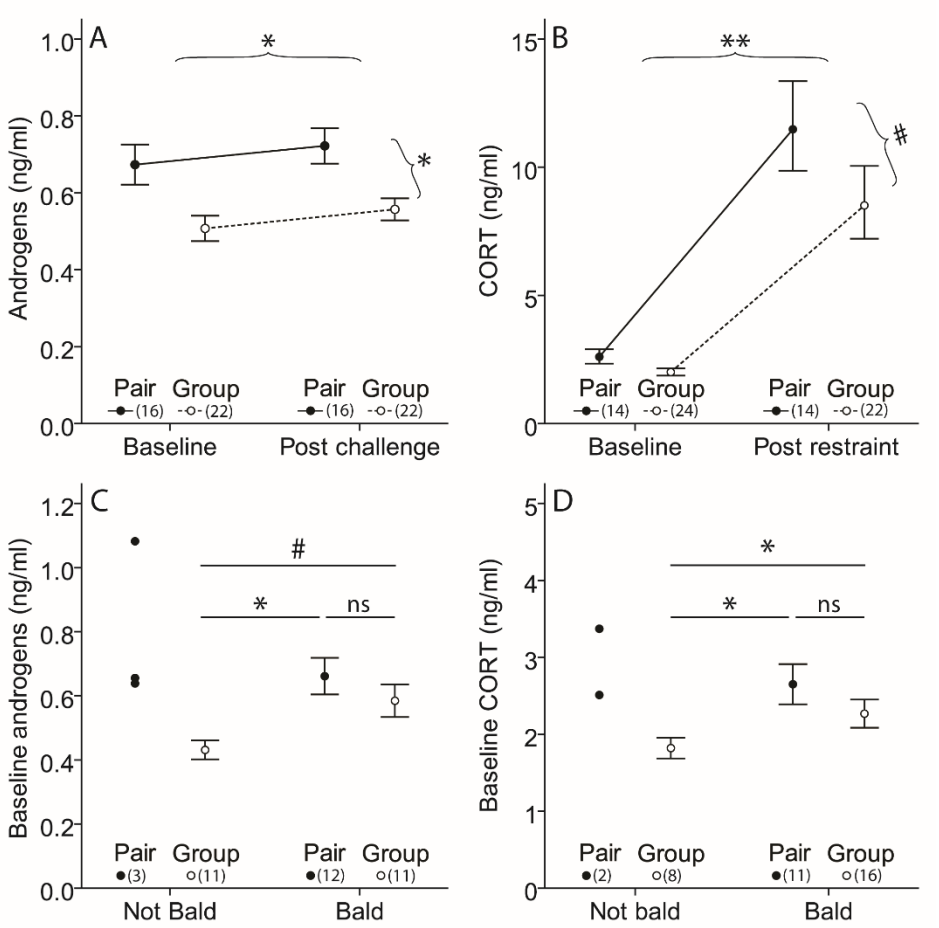

Figure 2.2. Plasma hormones. (A) plasma androgen concentration in ng/ml before and 30 minutes after an injection with 5 µg GnRH. (B) plasma CORT concentration in ng/ml before and after being restrained for 10 minutes (backtransformed from Log10). (C) baseline plasma androgen concentration and (D) baseline plasma CORT concentrations in ng/ml of bald and non-bald pair-housed females, and bald and non-bald group-housed females. Data from bald pair-housed females are indicated by solid circles, but were not used in the statistical analyses. Data are shown as means ± 1 SEM. Numbers between brackets indicate sample sizes. ** = $p < 0.01$; * = $p < 0.05$; # = $p < 0.1$; ns = not significant.

2.3.2. Corticosterone

Overall, pair-housed females had slightly higher plasma CORT concentrations than group-housed females, but the difference did not reach statistical significance ($F_{(1, 28.13)\ Treatment} = 3.61$, $p = 0.07$; table 2.1, Figure 2.2B). 10 minute restraint significantly increased plasma CORT concentrations ($F_{(1, 53.93)\ Sample} = 137.06$, $p < 0.01$; table 2.1, Figure 2.2B), but the increase did not differ between social treatments ($F_{(1, 52.90)\ Treatment * sample} = 0.02$, $p = 0.88$; Figure 2.2B). Individual baseline plasma CORT concentrations were not significantly correlated with baseline plasma androgen concentrations ($r_{(28)} = 0.23$, $p = 0.22$; Supplementary Figure 2.1A) and post-challenge plasma CORT concentrations did not correlate with post-challenge androgen levels ($r_{(27)} = 0.21$, $p = 0.26$; Supplementary Figure 2.1B).

2.3.3. Social proximity, baldness and baseline plasma hormones

In both pairs and groups, the proportion of time a female spent sitting with the male did not predict baseline plasma androgen or CORT concentrations, nor did the proportion of time a female spent sitting with at least one other female in her group (All F-values < 1.32, all corresponding p-values > 0.26; Supplementary Figure 2.2A-D).

Bald pair-housed females, bald group-housed females and non-bald group-housed females differed significantly in their baseline plasma androgen and CORT concentrations (baseline androgens: $F_{(2, 6.89)\ baldness} = 5.24$, $p = 0.04$; Figure 2.2C; baseline CORT: $F_{(2, 28.55)\ baldness} = 3.48$, $p = 0.045$; Figure 2.2D). Non-bald group-housed females had significantly lower baseline plasma androgen concentrations than bald pair-housed females and had marginally lower baseline androgen concentrations than bald group-housed females, although the latter was not statistically significant ($t_{(7.17)} = 3.21$, $p = 0.02$ and $t_{(5.64)} = 2.12$, $p = 0.08$ respectively). In addition, non-bald group-housed females had significantly lower baseline plasma CORT concentrations than both bald pair-housed females and bald group-housed females ($t_{(26.26)} = 2.52$, $p = 0.02$ and $t_{(29.13)} = 2.07$, $p = 0.048$ respectively). Baseline plasma androgen and CORT concentrations did not differ between bald pair-housed and bald group-housed females (baseline androgens: $t_{(9.07)} = 1.00$, $p = 0.35$; baseline CORT: $t_{(21.04)} = 0.85$, $p = 0.40$). Since there were only three non-bald pair-housed females that were still together with their male at the time of the hormone measurements, we did not include them in the statistical analysis. However, their androgen (n = 3) and CORT (n = 2) values were higher than most non-bald group-housed females and as high as most of the bald females in both social treatments (Figure 2.2C and 2.2D).

2.3.4. Yolk testosterone

We averaged yolk T values for each cage because eggs could not be assigned to individuals for group-housed females. Despite the treatment differences in plasma androgen concentrations, yolk T concentrations did not differ between the social treatments ($F_{(1, 21.49)\ Treatment} = 0.33$, $p = 0.57$; table 2.1), nor did total yolk T levels ($F_{(1, 22.99)\ Treatment} = 0.22$, $p = 0.64$; table 2.1). Neither average baseline plasma androgen concentrations nor the average response to GnRH predicted yolk T concentrations ($F_{(1, 18.00)\ Baseline\ T} = 0.28$, $p = 0.60$; $F_{(1, 18.00)\ \Delta T\ in\ response\ to\ GnRH} = 1.23$, $p = 0.28$; Supplementary Figure 2.3A and B). We could directly correlate female plasma androgen and yolk T concentrations only in pair-housed females, but analysis also showed no relationship between the two ($F_{(1, 13)\ Baseline\ androgens} = 0.01$, $p = 0.92$; $F_{(1, 13)\ \Delta\ androgens\ in\ response\ to\ GnRH} = 0.42$, $p = 0.53$; Supplementary Figure 2.3A and B).

2.3.5. Growth and reproductive performance

Female growth was not affected by the social environment ($F_{(1, 31.16)\ Age * treatment} = 1.05$, $p = 0.31$; table 2.1, Supplementary Figure 2.4). The first eggs were laid on day 41, and by day 58 all females were laying, but the onset of egg laying did not differ between the social treatments ($F_{(1, 28.27)\ Treatment} < 0.01$, $p > 0.99$; table 2.1). Egg laying rates were higher in pair-housed females compared to group-housed females, but not significantly so (treatment: $\chi^2_{(1)} = 3.62$, $p = 0.06$, Figure 2.3A). For all females, the number of eggs laid increased over time ((collection day + collection day^2 + collection day^3): $\chi^2_{(3)} = 529.30$, $p < 0.01$, Figure 2.3A), and there was no significant difference in this increase between the social treatments (treatment*(collection day + collection day^2 + collection day^3): $\chi^2_{(3)} = 4.57$, $p = 0.21$; Figure 2.3A).

Egg mass was not affected by social treatment (treatment: $\chi^2_{(1)} = 0.17$, $p = 0.68$; table 2.1, Figure 2.3B), but it increased significantly over time ((day+day^2+day^3): $\chi^2_{(3)} = 328.50$, $p < 0.01$; Figure 2.3B). The increase in egg mass did not differ between treatments (treatment*(day+day^2+day^3): $\chi^2_{(3)} = 3.04$, $p = 0.39$; Figure 2.3B). Treatment did not affect yolk mass in the subset of eggs collected for yolk hormone analysis ($F_{(1, 21.82)\ Treatment} = 0.13$, $p = 0.72$; table 2.1).

Pair-housed females on average laid almost one more fertilized egg per female than group housed females, but the difference in fertility did not reach statistical significance ($z = -1.72$, $p = 0.09$; table 2.1, Figure 2.3C). Hatching success (the percentage of all eggs collected for the F1 generation that hatched, i.e. including infertile eggs) did not differ between treatments ($z = -1.16$, $p = 0.25$; table 2.1, Figure 2.3C). Hatchlings did not differ in body mass ($F_{(1, 11.45)\ Treatment} = 0.02$, $p = 0.91$; table 2.1) or tarsus length ($F_{(1, 16.02)\ Treatment} = 0.36$, $p = 0.56$; table 2.1).

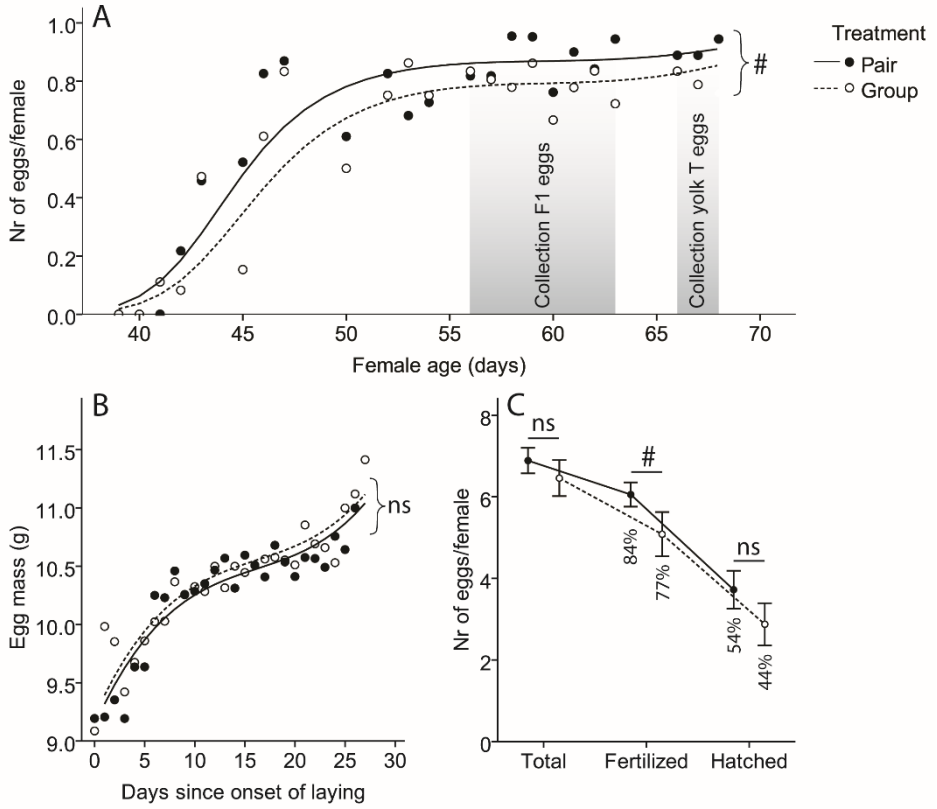

Figure 2.3. Measures of reproductive performance. (A) Egg production for both social treatments. Circles show the mean number of eggs/female found on a given day. Lines show the model predictions. n = 361 eggs from 23 pairs and 533 eggs from 12 groups. (B) Egg mass for both social treatments. Circles show the mean egg mass, lines the model predictions. n = 324 eggs from 23 pairs and 531 eggs from 12 groups. (C) Average number of eggs collected for F1 and the number and percentage of eggs that were fertilized and hatched, per social treatment. n = 124 eggs from 18 pairs, 155 eggs from 8 groups. Error bars indicate ± 1 SEM. * = $p < 0.05$; # = $p < 0.1$; ns = not significant.

2.4. Discussion

In many vertebrates, the social environment affects physiology and behaviour with consequences for female reproductive performance and offspring quality, but the underlying mechanisms are not well understood. We therefore investigated how the social environment - pair or group housing - of Japanese quail females affects their reproductive and stress physiology, yolk hormone deposition, and fecundity. Contrary to our expectations, female quail housed in pairs had higher plasma androgen concentrations and slightly higher CORT concentrations than females housed in groups, although the latter did not reach statistical significance. Treatment did not affect the HPG-axis or HPA-axis response to standardized challenges, nor were there differences in yolk T levels or fecundity. Female body mass, hatchling numbers, weight, and size were not affected by the social environment. Because baseline CORT levels were similar to plasma CORT concentrations found before in quail (Correa et al., 2011; Hayward et al., 2006; Marasco et al., 2012; Rutkowska et al., 2011) and females responded to the stress protocol with a

significant increase in CORT, it is unlikely that our birds had experienced chronic stress, potentially masking any treatment effects.

Previous studies found increased plasma androgen and CORT concentrations in females exposed to increased social stimulation, higher social density or social instability (Eisenegger et al., 2011; Guibert et al., 2010; Langmore et al., 2002; Mazuc et al., 2003; Nephew and Romero, 2003). We suggest that we found the opposite because of differences in the adult sex ratio between the social treatments, leading to differences in male-female interactions. In the present study, the males' attention in group cages was divided among three different females, whereas in pairs there was only one female to interact with. As a consequence, the effect of the male's presence on circulating female androgen and CORT concentrations was likely to be stronger in pairs than in groups. Indeed, previous studies have shown that the sex composition of groups (Campo and Davila, 2002), male courtship song (Marshall et al., 2005) and copulation with a male (Correa et al., 2011) can affect a female's endocrine status, including androgen and CORT levels. Higher male:female ratios induced stress in groups of domestic chicken (Campo and Davila, 2002), and male courtship song increased female circulating androgen levels in canaries (Marshall et al., 2005). In Japanese quail, copulation with a male increased female CORT levels, while the number of mounts by the male and male body condition positively correlated with the female's androgen response to copulation (Correa et al., 2011). Japanese quail males often engage in forced copulations, which has been suggested as a source of stress for females (Adkins-Regan, 2015, 1995; Galef Jr., 2008; Rutkowska et al., 2011). Interestingly, in the group treatment, bald females, who presumably experienced more copulation attempts by the male, had higher baseline androgen and CORT levels than non-bald females and levels that were more similar to those of pair-housed females. In addition, there were hardly any non-bald females in pairs. This supports the idea that the intensity of male-female interactions is an important factor affecting female circulating hormone concentrations. However, it remains to be investigated whether the results found in the present study were indeed due to an effect of copulation with the male or due to other aspects of the treatment. In addition, the social environment most likely affects male endocrine status as well, which may have important consequences for male-female interactions (Ball and Balthazart, 2008; Cheng and Lehrman, 1975; Erickson, 1970) and should be taken into account in future studies.

Effects of the adult sex ratio may not be solely due to an influence of males, but also due to the number of females present. Female Japanese quail prefer to associate with other females when a male is present, indicating that females may try to avoid unwanted sexual attention by the male by grouping together (Persaud and Galef Jr., 2003). In the present study, the effects of the male may therefore be alleviated in groups due to social buffering, although we did not find a correlation between female-female social proximity and baseline hormone levels.

Although we did not find an effect of social proximity on hormone levels, we cannot rule out potential effects of cage size. Since pairs were housed in cages that were 50% smaller than cages for groups to ensure a comparable social density between treatments,

the differences in cage size may have affected individual movement patterns and use of space (Leone and Estevez, 2008) and thereby social interactions and social avoidance behaviour in particular.

Several studies in birds have shown that breeding density, social instability, social motivation and female-female competition is positively correlated with yolk androgen concentrations, yolk T in particular (Bentz et al., 2013; Eising et al., 2008; Guibert et al., 2010; Hargitai et al., 2009; Mazuc et al., 2003; Mills and Faure, 1991; Pilz and Smith, 2004; Reed and Vleck, 2001; Schwabl, 1997; Whittingham and Schwabl, 2002). In contrast, we did not find effects of group size on average yolk T in the present study. Social instability did lead to increased yolk androgen levels in a previous study on Japanese quail (Guibert et al., 2010). We expected a similar effect in group-housed females because we assumed that group living would result in a less stable social environment than pair housing since females would encounter and interact with multiple individuals. However, groups may have been less stable than pairs only at the start of the social treatments when group-housed individuals had to familiarize themselves with more conspecifics than pair-housed birds. Since the social environment remained stable over time in both treatments and eggs for yolk T measurements were collected 37 days after the start of the social treatments, the females likely had ample time to familiarize with their group members, and any initial differences in yolk T levels might have disappeared over the course of the experiment. Finally, variation between females within groups may have masked treatment differences in yolk T concentrations, which should be analysed in future studies as we unfortunately could not assign eggs to individual females in groups.

The magnitude of the plasma androgen response to GnRH has been proposed to be a better predictor of yolk androgen deposition than baseline plasma androgen concentrations (Jawor et al., 2007; Müller et al., 2011). However, this has only been investigated by correlating a female's androgen response to GnRH with yolk androgen levels measured in subsequently laid eggs (Jawor et al., 2007; Müller et al., 2011; Peluc et al., 2012). In the present study, we assessed the female's androgen response to GnRH during egg laying, but after we had collected eggs for yolk hormone measurement. Neither baseline plasma androgens nor the androgen response to GnRH predicted yolk T levels, suggesting that yolk T does not reflect the female's inherent sensitivity to GnRH, but rather that stimulation by GnRH may affect yolk T deposition in eggs laid later. Another possibility is that the link between the GnRH response and yolk T may be dosage-dependent and context-dependent (Jawor et al., 2007). We found a significant increase in plasma androgen levels in response to GnRH injections, but the increase was smaller than that found in previous studies with Japanese quail using similar dosages of chicken GnRH-I (Peluc et al., 2012). This might be due to genetic or environmental differences between populations and studies, and a low response to GnRH may not be reflected in yolk T levels, as opposed to a high GnRH responsiveness.

Female reproductive performance was largely unaffected by the social treatments, indicating that the effects on female's endocrine physiology had little fitness consequences. A possible explanation is that the differences in social treatments had little effect since

domesticated Japanese quail have been heavily selected for egg production (Adkins-Regan, 2015; Cheng et al., 2010; Mills et al., 1997; Nichols, 1991; Schmid and Wechsler, 1997). Moreover, since only a subset of eggs was incubated to calculate fertility and number of hatchlings, these differences might have been larger had all eggs been incubated. Although the difference did not reach statistical significance, it is noteworthy that pair-housed females had somewhat higher egg laying rates and fertility compared to group-housed females. Egg production and fertility may be slightly higher in pairs because the higher male:female ratio may have stimulated female reproduction (Bentley et al., 2000; Brockway, 1965; Erickson, 1970), as demonstrated by the fact that most pair-housed females were bald and therefore experienced a higher rate of copulation. In addition, pair-housed quail might have had higher levels of within-pair testosterone covariation which has been found to positively predict reproductive output (Hirschenhauser et al., 2010, 1999; but see Hirschenhauser, 2012).

2.5. Conclusion

Contrary to expectations, we found that increased group size did not result in elevated plasma androgen or CORT concentrations. Instead, we found higher circulating androgen and CORT levels in pair females, possibly due to a stimulating effect of a higher frequency and intensity of copulations with the male on female physiology. These treatment effects were not reflected in yolk T levels, and in contrast to previous studies the plasma androgen response to GnRH was not correlated with yolk T, suggesting independent regulation of plasma hormones and yolk hormones. In addition, there were no strong effects on reproductive performance. The unexpected finding of higher circulating androgen and CORT levels in pair-housed females demonstrate that we need a better understanding of how group sex ratios and specific aspects of male-female and female-female relationships and their interactions affect female endocrine physiology.

Acknowledgements

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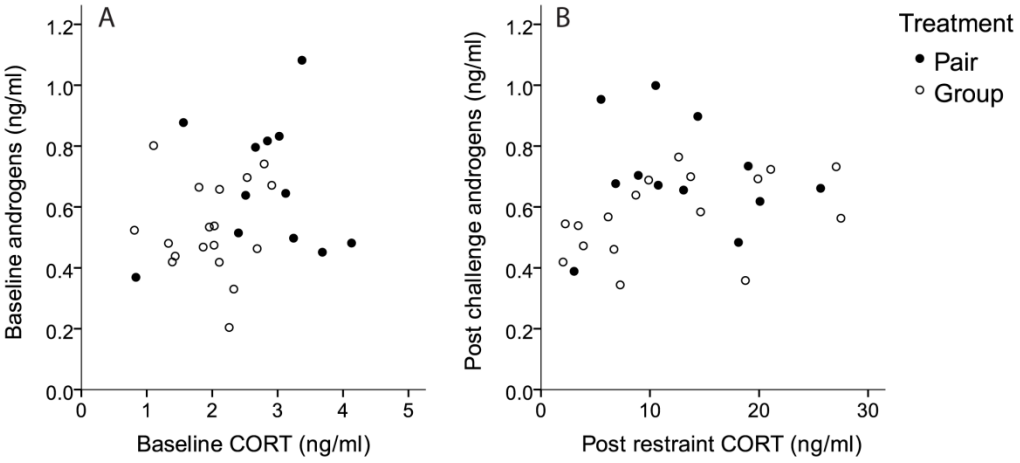
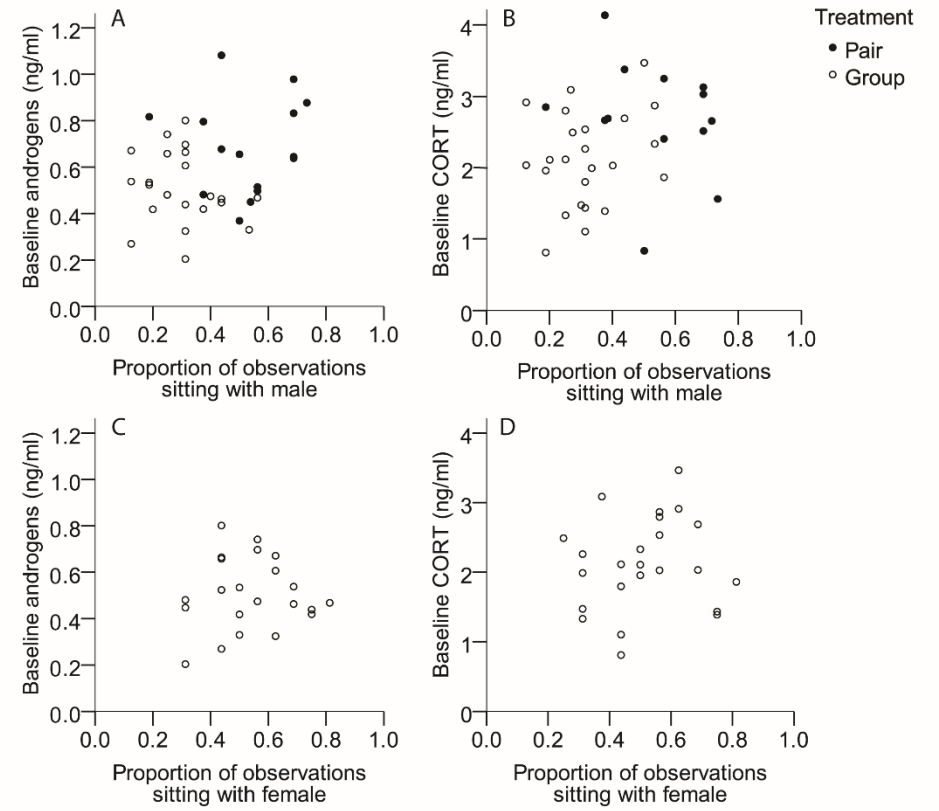
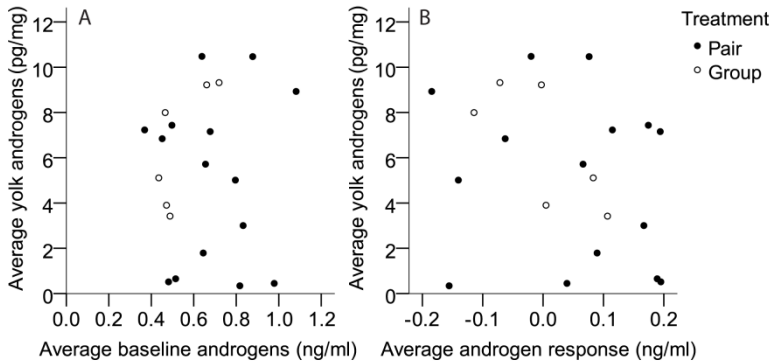
Supplementary material



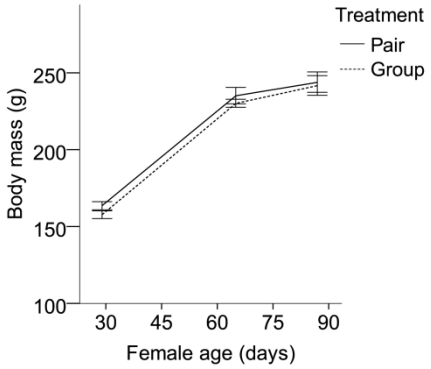
Supplementary Figure 2.1. Correlations between baseline and post challenge concentrations of androgens and CORT in female plasma. (A) Individual baseline plasma CORT concentrations plotted against baseline plasma androgen concentrations, and (B) individual post-challenge plasma CORT concentrations plotted against post-challenge androgen levels for both social treatments. Neither baseline nor post-challenge CORT and androgen concentrations were correlated with each other.



Supplementary Figure 2.2. Social proximity and baseline plasma hormones. The proportion of all scan observations that a female was found sitting within a distance of one body-length from the male, plotted against the (A) baseline androgen concentration and the (B) baseline CORT concentration in her plasma, for both social treatments. The proportion of time a female spent sitting with the male did not predict baseline plasma CORT or androgen concentrations (CORT: $F_{(1, 33.60)\ \text{Sitting with male}} = 0.02$, $p = 0.88$; androgens: $F_{(1, 33.45)\ \text{Sitting with male}} = 0.11$, $p = 0.75$), and this was true for both social treatments (CORT: $F_{(1, 32.95)\ \text{Treatment*sitting with male}} = 1.32$, $p = 0.26$; androgens: $F_{(1, 32.40)\ \text{Treatment*sitting with male}} = 0.45$, $p = 0.51$). (C) and (D) show the proportion of scan observations that a group-housed female was found sitting close to another female, plotted against (C) her baseline plasma androgen concentration and (D) her baseline plasma CORT concentration. The proportion of time a female spent sitting with at least one other female did not predict baseline plasma CORT or androgen concentrations (CORT: $F_{(1, 20.98)\ \text{Sitting with female}} = 0.26$, $p = 0.62$; androgens: $F_{(1, 17.07)\ \text{Sitting with female}} = 0.25$, $p = 0.63$).



Supplementary Figure 2.3. Correlations between plasma androgens and yolk T. (A) Average baseline plasma androgen concentrations per cage and (B) the average androgen response to GnRH per cage plotted against the average yolk T concentrations per cage. Note that in pair housed-females, the average concentrations are the same as the individual female's concentrations as there is only one female per cage. For group-housed females, hormone values were averaged per cage because eggs could not be assigned to individuals within groups. Neither baseline plasma androgen concentrations, nor the response to GnRH predicted yolk T concentrations.



Supplementary Figure 2.4. Female growth between day 65 and day 87. Depicted is the average body mass of both pair-housed and group-housed females, which did not differ between treatments, on day 19 (n = 24 pair-housed females and 36 group-housed females from 12 groups), 65 (n = 17 pair-housed females and 33 group-housed females from 11 groups) and day 87 (n = 13 pair-housed females and 12 group-housed females from 4 groups). Error bars indicate ± 1 SEM.

Supplementary Data 2.1. Raw data; available at https://doi.org/10.1371/journal.pone.0176146.s005

Chapter 3

No evidence for sex-specific effects of the maternal social environment on offspring development in Japanese quail (*Coturnix japonica*)

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Abstract

The social environment of reproducing females can cause physiological changes, with consequences for reproductive investment and offspring development. These prenatal maternal effects are often found to be sex-specific and may have evolved as adaptations, maximizing fitness of male and female offspring for their future environment. Female hormone levels during reproduction are considered a potential mechanism regulating sex allocation in vertebrates: high maternal androgens have repeatedly been linked to increased investment in sons, whereas high glucocorticoid levels are usually related to increased investment in daughters. However, results are not consistent across studies and therefore still inconclusive. In Japanese quail (*Coturnix japonica*), we previously found that pair-housed females had higher plasma androgen levels and tended to have higher plasma corticosterone levels than group-housed females. In the current study we investigate whether these differences in maternal social environment and physiology affect offspring sex allocation and physiology. Counter to our expectations, we find no effects of the maternal social environment on offspring sex ratio, sex-specific mortality, growth, circulating androgen or corticosterone levels. Also, maternal corticosterone or androgen levels do not correlate with offspring sex ratio or mortality. The social environment during reproduction therefore does not necessarily modify sex allocation and offspring physiology, even if it causes differences in maternal physiology. We propose that maternal effects of the social environment strongly depend upon the type of social stimuli and the timing of changes in the social environment and hormones with respect to the reproductive cycle and meiosis.

3.1. Introduction

Variation in the social environment affects female behaviour and physiology with potential consequences for reproductive investment. Changes in reproductive investment, in turn, modify the prenatal environment of the developing offspring and can thereby profoundly shape offspring's future phenotype (Groothuis et al., 2005; Guibert et al., 2010; Kaiser and Sachser, 2009, 2005). Pre- and postnatal maternal effects of the social environment can bias offspring sex ratios (Clutton-Brock and Iason, 1986; Michler et al., 2013; Minias et al., 2014) and affect offspring development and behaviour, often in a sex-specific way (Kaiser and Sachser, 2009, 2005). Sex-specific maternal effects are thought to have evolved as adaptations, maximizing fitness of male and female offspring for their anticipated environment. Maternal steroid hormones provide important candidate signals, transmitting effects of the social environment across generations. Hormones, and other compounds, are transferred to the ovum and embryo and can profoundly affect offspring behavioural and physiological development (Groothuis et al., 2005; Groothuis and Schwabl, 2008; Kaiser and Sachser, 2005; Meylan et al., 2012; Radder, 2007; von Engelhardt and Groothuis, 2011).

Oviparous species, such as reptiles and birds, are especially suitable to explore prenatal effects because the maternal and offspring environment can be independently manipulated. Previous studies on avian species have shown effects of the social environment on sex allocation (Michler et al., 2013; Minias et al., 2014). In the great cormorant (*Phalacorcorax carbo sinensis*), social density positively correlates with the proportion of male offspring within broods (Minias et al., 2014). In contrast, female great tits (*Parus major*) bred in areas with experimentally increased nesting densities or who experienced areas with high nesting density as juveniles produce female-biased broods in the following year, whereas females breeding in areas with decreased nesting densities or reared in areas of naturally lower nesting density produce male-biased broods (Michler et al., 2013). In many vertebrate species, changes in both primary and secondary offspring sex ratio have been linked to variation in maternal plasma steroids around conception (reviewed by Alonso-Alvarez, 2006; James, 2008; Krackow, 1995; Navara, 2013a; Pike and Petrie, 2003). In avian species, increased levels of maternal androgens usually lead to male-biased offspring sex ratios (Goerlich-Jansson et al., 2013; Goerlich et al., 2009; Pike and Petrie, 2005; Rutkowska and Cichoń, 2006; Veiga et al., 2004, but see Correa et al., 2011), whereas increased levels of maternal glucocorticoids often result in female-biased offspring sex ratios (Bonier et al., 2007; Goerlich-Jansson et al., 2013; Love et al., 2005; Pike and Petrie, 2006, 2005, but see Gam et al., 2011; Henriksen et al., 2013). In Japanese quail (*Coturnix japonica*), naturally increased maternal faecal corticosterone (CORT) metabolite concentrations and experimentally elevated maternal plasma CORT concentrations are associated with a female-biased primary sex ratio (Pike and Petrie, 2006). In contrast to the findings in other species, maternal plasma testosterone levels of Japanese quail have been related to both an unbiased (Pike and Petrie, 2006) as well as a female-biased offspring sex ratio (Correa et al., 2011). This indicates that results from experimental and correlational studies are still inconclusive.

In addition to affecting offspring sex ratio, the maternal social environment can affect offspring growth and survival, which may be mediated by changes in maternal circulating levels of androgens and CORT. For example, in American red squirrels (*Tamiasciurus hudsonicus*), increased offspring growth rates at higher social densities have been attributed to the effects of increased maternal CORT (Dantzer et al., 2013). In Japanese quail, social instability resulted in an increase in agonistic interactions and reduced offspring body mass at the age of 1-3 weeks, compared to stable social groups (Guibert et al., 2010). Such effects on growth may be due to increased maternal CORT because artificially increasing maternal circulating CORT reduced offspring growth in Japanese quail (Hayward and Wingfield, 2004). The maternal social environment and maternal hormones can also have sex-specific effects on offspring growth and survival. In guinea pigs (*Cavia aperea*), housing females individually during pregnancy decreased growth of daughters compared to daughters of group-housed females, whereas growth of sons was non-significantly increased (von Engelhardt et al., 2015). Artificially increasing maternal circulating testosterone in zebra finches (*Taeniopygia guttata*) reduced the hatching success of sons and increased the post-hatching survival of daughters (Rutkowska and Cichoń, 2006). Experimental elevation of maternal CORT in European starlings (*Sturnus vulgaris*) increased the mortality of male embryos, led to a female-biased sex ratio at hatching, and reduced early growth in males (Love et al., 2005).

Maternal effects on offspring growth and survival may be attributed to (sex-specific) modulation of offspring endocrine physiology (Groothuis et al., 2005; Groothuis and Schwabl, 2008; Kaiser and Sachser, 2005) since both growth and survival can relate to circulating hormone levels (e.g. Braasch et al., 2011; Brown et al., 2005; Goodship and Buchanan, 2006; Goutte et al., 2010; Groothuis and Ros, 2005; Hull et al., 2007; Müller et al., 2009; Ros, 1999; Wada and Breuner, 2008). Studies on transgenerational effects of the maternal social environment on offspring physiology are scarce, especially in birds. However, in Japanese quail, maternal social instability increases the offspring's emotional reactivity scored in different behavioural tests, suggesting possible effects on the hypothalamic–pituitary–adrenal axis (HPA-axis) regulating the release of CORT (Guibert et al., 2010). This assumption is corroborated by studies on guinea pigs, which even find sex-specific effects of the maternal social environment on the HPA-axis in the offspring (Kaiser and Sachser, 2001; von Engelhardt et al., 2015).

In our previous study on Japanese quail, we have shown that the social environment during breeding affects female physiology (Langen et al., 2017). Females housed in pairs (one male, one female) had higher plasma androgen concentrations and tended to have higher plasma CORT concentrations than females housed in groups (one male, three females; see Langen et al. 2017 for more details). Here, we examined the offspring of those females to investigate whether the maternal social environment affects offspring sex ratio and has sex-specific effects on mortality, growth and endocrine physiology. We expected overall positive effects on daughters of pair-housed females, i.e., a bias towards female offspring because higher maternal androgen (Correa et al., 2011) and CORT levels (Pike and Petrie, 2006) have been linked to a female-biased offspring sex ratio

in Japanese quail. Furthermore, we expected increased growth and decreased mortality in daughters of pair-housed mothers because elevated maternal plasma androgen or CORT levels had positive effects on daughters and negative effects on sons in other avian species (Love et al., 2005, Rutkowska and Cichoń, 2006). In contrast to female-biased reproductive investment of pair-housed mothers, we expected a potentially male-biased offspring sex ratio, increased growth and decreased mortality in sons of group-housed mothers. We also investigated whether offspring from pair-housed and group-housed mothers differ in their circulating androgen levels and the sensitivity of the HPA-axis.

3.2. Materials and methods

3.2.1. Ethics statement

All experimental procedures and humane endpoints for minimizing suffering were approved by the North Rhine-Westphalia State Agency for Nature, Environment and Consumer Protection (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen), Recklinghausen, Germany (licence number 84-02.04.2013-A127). Animal facilities were approved for keeping and breeding Japanese quail for research purposes by the local government authority responsible for health, veterinary and food monitoring (Gesundheits-, Veterinär- und Lebensmittelüberwachungsamt Bielefeld, Germany).

3.2.2. Parental generation

The parental generation originated from eggs generously provided by the INRA in Nouzilly, France (Experimental unit 1295 (UE PEAT) and UMR 85, Physiologie de la Reproduction et des Comportements, INRA-CNRS-IFCE-Université de Tours, Val de Loire Center, Nouzilly, France). These eggs were produced by females from a non-selected control line, bred next to quail lines selected for low or high social reinstatement (Mills and Faure, 1991). They were incubated and reared at Bielefeld University (Germany) and placed into their social treatments at 29 days of age. The social treatment was either pair-housing or group-housing, with groups (n=12) consisting of three females and one male, pairs (n=24) of one female and one male. Siblings or half siblings were never housed in the same cage. The birds were kept indoors, in two adjacent rooms with artificial lighting and no natural daylight. The light-dark cycle was 14:10h, and the temperature was set to 20°C. Pair cages measured 75 x 80 x 40 cm, group cages 150 x 80 x 40 cm. The distribution of the cages across and within rooms was balanced across treatments. The birds were kept on wood shavings, and all cages contained a sand bath and one shelter hut per female. Food (GoldDott Hennenmehl, Derby Spezialfutter GmbH, Münster, Germany) and water was provided ad libitum. On a weekly basis, the standard diet was supplemented with mealworms and shell grit. After collecting eggs for breeding the next generation, the parental females were tested for their hormonal response to a stressor (at 66-67 days of age; see Figure 3.1) and to an injection with gonadotropin releasing hormone (at 72 days of age; see Figure 3.1), and we measured growth, reproductive output, and egg yolk testosterone concentrations (Langen et al., 2017).

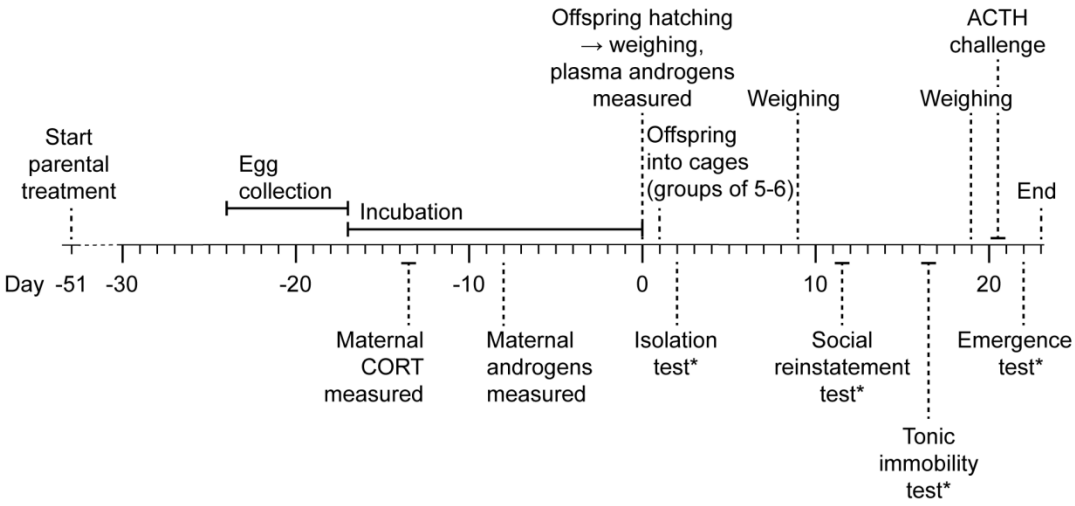


Figure 3.1. Timeline of experimental procedures. Procedures marked with * are behavioural tests which are not reported here.

3.2.3. Egg incubation and hatching

After the parental generation had been housed in their treatment groups for 27 days (at 8 weeks of age), eggs (124 eggs from pair-housed females and 155 eggs from group-housed females) were collected over the course of one week, weighed to the nearest 0.1 g, and incubated to produce the offspring generation. Eggs were incubated in a HEKA-Euro-Lux II incubator (HEKA-Brutgeräte, Rietberg, Germany) in complete darkness to avoid the effects of light on development and because it more likely reflects the situation during natural incubation (Archer and Mench 2014). Until incubation day 14, the temperature was set at 37.8°C, humidity to 55%, and the eggs were turned every 2 hours. After 9 days of incubation, the eggs were candled to identify embryonic development and non-fertilized eggs were removed (remaining eggs: 107 eggs from pair-housed females and 121 eggs from group-housed females, Table 3.1). On day 15 of incubation, eggs were moved to hatching trays, the incubation temperature was set to 37.5°C, the humidity to 75%, and the eggs were no longer turned. The hatching trays were divided into separate compartments (5.5 x 5.5 x 5 cm) for each individual egg so that we could identify which chick hatched from which egg. The compartment walls were made of transparent Plexiglas and the bottom of each hatching tray was made of mesh wire, allowing air flow and olfactory and acoustic communication between the chicks. The offspring hatched after 17 ± 1 days of incubation. Some hatchlings were excluded from the experiment because they had birth defects (two male offspring from two pair-housed mothers, one female offspring from one group-housed mother). In addition, some offspring originated from cages in which birds had to be separated before or during egg collection due to aggression (two male and one female offspring from two mothers housed in the same group and six male and six female offspring from two of five separated pairs; see Langen et al., 2017 for more information). These offspring from separated parental cages were not included as subjects in the present study but used as cage mates. A total of 35 male and 29 female offspring from pair-housed

mothers, and 34 male and 33 female offspring, and one hatchling of unidentified sex from group-housed mothers remained (Table 1).

Birds were removed from the incubator once their feathers had dried (ca. 2 hours after hatching), weighed to the nearest 0.1 g and their tarsus was measured to the nearest mm using a digital caliper. To measure circulating androgen levels at hatching and to assign genetic sex and parentage, a small blood sample (max. 50 µl, or about 0.7% of body weight which does not appear to have long-term effects on adult or developing birds; Sheldon et al. 2008) was taken by piercing the jugular vein with a sterile 27 gauge needle and collecting the blood in heparinized capillaries (BRAND GMBH + CO KG, Wertheim, Germany). As we were unable to retrieve blood from 25 out of 132 chicks, a piece of egg shell membrane (ca. 2 x 2 mm) containing blood vessels was collected for genetic sex and parentage assignment. From the remaining 107 chicks, we were able to retrieve at least a small amount of blood for DNA extraction, and 53 of these samples were further used for androgen measurements (Table 1).

After 19 days of incubation, all eggs that had not hatched were removed from the incubator and a tissue sample was taken from dead embryos for genetic sex determination.

3.2.4. Offspring husbandry

After weighing and measuring, the offspring were all kept together for the first night in a 100 x 80 x 80 cm cage on waved cardboard and with two heating lamps and food (ground pellets: GoldDott Enten-Gänsestarter - no coccidiostat, Derby Spezialfutter GmbH, Münster, Germany) and water provided ad libitum. Lights remained on for the first night. The next day, the birds were placed into smaller groups of five to six unrelated individuals (all from the same parental treatment, n = 14 cages of pair offspring, n = 12 cages of group offspring). At that time, the offsprings' sexes were still unknown, therefore the chicks were randomly allocated across groups. Offspring cages measured 75 x 80 x 40 cm, contained heating pads partially covered by a small hut (15 x 13 x 13 cm), and ad libitum water and food. The main lights were set to a 14:10h light-dark cycle (lights on at 5 am), but small night lights were placed approximately 1 m in front of the cages to make sure the birds were able to find food and water during the night. Birds were kept on waved cardboard until 8 days post-hatching, after which they were kept on wood shavings.

All cages were checked daily, and we recorded whether any of the birds had died to be able to measure differences in mortality between offspring from the two maternal treatments. To analyse offspring growth, all birds were weighed to the nearest 0.1 g on the day of hatching, and on post-hatching days 9 and 19. Between the day of hatching and day 23, the offspring underwent several behavioural tests, the results of which will be described elsewhere (Langen et al. in prep). On post-hatch day 20-21, we assessed the birds' CORT response to an injection with adrenocorticotropic hormone. For a timeline of all experimental procedures, see Figure 3.1. Sample sizes per measure vary (Table 3.1) since some of the offspring died in the first few weeks or because we were unable to get enough plasma for the physiological measurements.

Table 3.1. Samples sizes for each measurement.

	Offspring from pair-housed mothers				Offspring from group-housed mothers			
Measure	**Total**	**Sons**	**Daughters**	**Mothers**	**Total**	**Sons**	**Daughters**	**Mothers (groups)**
Sex ratio								
Primary	107[1]	49	47	18	121[1]	53	56	- [2] (8)
At hatching	64	35	29	17	68[1]	34	33	20 (8)
Correlation with maternal androgens	59	33	26	15	49	27	22	15 (6)
Correlation with maternal CORT	47	23	24	12	49[1]	25	23	16 (7)
At day 23	54	27	27	16	56	28	28	17 (7)
Mortality								
Overall	64	35	29	17	68[1]	34	33	20 (8)
Correlation with maternal androgens	59	33	26	15	49	27	22	15 (6)
Correlation with maternal CORT	47	23	24	12	49[1]	25	23	16 (7)
Egg mass	64	35	29	17	68[1]	34	33	20 (8)
Mass at hatching	64	35	29	17	68[1]	34	33	20 (8)
Mass at day 9	56	28	28	17	57	28	29	19 (8)
Mass at day 19	54	27	27	16	56	28	28	17 (7)
Hatchling androgens								
Individual samples	17	13	4	16	12	5	7	13 (7)
Pools	7	4 (8)[3]	3 (7)[3]	16	4	2 (4)[3]	2 (5)[3]	13 (7)
ACTH challenge								
Baseline	384	224	164	154	484	264	224	16 (8)[4]
Response	374	224	154	154	474	264	214	16 (8)[4]

[1] Sexing was unsuccessful in 23 embryos and 1 hatchling. [2] In groups, mothers of embryos were not identified. [3] Number of individuals included in the plasma pool. [4] Reduced sample sizes due to insufficient plasma for the CORT analysis

3.2.5. ACTH challenge

In order to test the offspring's HPA-axis sensitivity, we measured the plasma CORT increase following an injection of adrenocorticotropic hormone (ACTH, which stimulates glucocorticoid production in the adrenal glands and is normally released by the pituitary in response to corticotrophin-releasing hormone from the hypothalamus) on post-hatch day 20-21. All birds were tested between 09:00 am and 1:00 pm, and plasma CORT levels did not change significantly during that period ($\chi^2_{(1)}$: 1.55, p = 0.21).

For the ACTH challenge, all birds from one cage were caught and transported to the experimental room in a transport box (40 x 30 x 40 cm). A blood sample was taken to determine baseline plasma CORT concentrations by puncturing the ulnar vein with a sterile 27 gauge needle and collecting 200-300 µl blood in heparinised capillaries (BRAND GMBH + CO KG, Wertheim, Germany). We recorded the time between opening the cages and taking the baseline blood sample (range: 71-287 seconds, mean ± SEM: 155 ± 6 seconds).

After the baseline blood sample was taken, the birds were injected in the pectoral muscle with 0.8 µg ACTH (H-1150.0001, Bachem, Bubendorf, Switzerland) dissolved in 50 µl PBS (average dosage ca. 10 µg/kg) and placed back in the transport box. 10 minutes post injection, the birds were caught again, and a second blood sample was taken to determine the CORT response to ACTH.

3.2.6. Hormone analysis

After blood samples were taken to determine androgen (at the day of hatching) and CORT levels (in the ACTH challenge), samples were kept on ice for a maximum of two hours and then centrifuged for 10 minutes at 2000 x g. The plasma was then collected and frozen at -20°C for future use.

We used a commercial testosterone ELISA Kit (Demeditec Diagnostics GmbH, Kiel, Germany, cat. no. DES6622) to determine plasma androgen concentrations. Cross reactivity of the kit antibody, as reported by the manufacturer, was 23.3% for 5α-Dihydrotestosterone, 1.6% for Androstenedione, and less than 0.1% for other steroids. Samples were distributed over two assays, balanced for maternal treatment. The inter-assay coefficient of variation (CV) was 1.64% (based on two quail plasma pools measured in each assay). Since we were unable to get sufficient plasma from 24 out of 53 chicks, the 24 samples were pooled by combining samples from two to three hatchlings in each pool, resulting in 11 plasma pools. We pooled plasma samples from hatchlings within the same sex and maternal treatment and, where possible, pools consisted of samples from full siblings. In total, 40 samples were measured in the androgen assay (29 single plasma samples and 11 plasma pools; Table 3.1). In four out of the 40 samples (two from sons of pair-housed mothers, two from daughters of group-housed mothers), androgen concentrations were below the range that could be estimated using the standard curve and were therefore assigned the lowest measured value (28.2 pg/ml), as a conservative estimate.

Plasma CORT concentrations in the ACTH challenge were determined using a commercial Corticosterone RIA Kit (MP Biomedicals, Orangeburg, USA, cat. no. 07-102102). Cross reactivity of the kit antibody, as reported by the manufacturer, was 0.34% for Desoxycorticosterone, 0.1% for Testosterone, and less than 0.1% for other steroids. Samples were measured together with quail plasma samples from other experiments and distributed over 11 assays, balanced for treatment. The intra-assay CV was 4.51%, the inter-assay CV was 13.86% (based on a chicken plasma pool and two kit controls measured in each assay). 170 samples were measured in the CORT assay (86 baseline, 84 post-ACTH; Table 3.1), and in 15 cases the CORT values were above the highest assay standard (all post-ACTH samples, from eight sons of pair-housed mothers and seven sons of group-housed mothers). As we were unable to repeat measurements at a higher dilution these samples were assigned a value of 35 ng/ml (based on the value of the highest assay standard) as a conservative estimate.

3.2.7. Genetic sex and parentage assignment

We used molecular methods to determine offspring sex and to assign parentage of all hatched offspring to one of the three potential mothers in the group treatment. The concentrated blood cells left over after centrifuging blood for hormone measurements were diluted 1:2 with phosphate buffer saline (10 mM PBS+6 mM EDTA, pH 7.4) and stored at -20°C. Similarly, tissue samples from non-hatched embryos were frozen at -20°C for future use. Genomic DNA was obtained by a phenol/chloroform or Chelex extraction (Walsh et al., 1991). Genetic sex determination was then performed using primers 2550f and 2718r (Fridolfsson and Ellegren, 1999).

We genotyped offspring and parents at 22 microsatellite loci using fluorescently labelled primers, as described previously (Langen et al., 2017). Parentage was then manually assigned by identifying which genotype of the three potential mothers in a cage best matched the offspring genotype.

3.2.8. Statistics

All statistical analyses were done using the lme4 package (Bates et al., 2015) of R 3.2.3 (R Core Team, 2015).

To analyse the effect of the maternal social environment and maternal hormones on offspring sex ratio and mortality, generalized linear mixed models with a binomial distribution and logit link function were fitted. Models included the maternal social environment as a fixed effect. Additionally, models of offspring mortality included a fixed effect of offspring sex and its interaction with the maternal social environment. We tested for a sex-ratio bias in each of the maternal social environments, where a significant effect of the intercept on the logit scale indicates a deviation from parity. Finally, we tested for non-random (extra-binomial) variance of sex-ratios using simulations (see Postma et al. 2011). We generated a distribution of 1000 expected clutch sex ratios based on the observed mean sex ratio and the number of offspring from each mother or each maternal

cage (for embryos whose parentage was not assigned) and compared whether the observed variance in sex-ratios fell outside the upper confidence interval (overdispersion) or lower confidence interval (underdispersion) of the simulated data. We then analysed the effects of maternal hormones on offspring sex ratio and mortality using separate models, either with maternal baseline plasma androgen or with baseline plasma CORT levels as fixed effects. Maternal treatment was not included in these models to avoid multicollinearity because maternal hormones differed according to maternal treatment.

General linear mixed models were used to analyse the effect of the maternal social environment and offspring sex on egg mass, offspring mass and offspring circulating hormone levels (androgens at hatching and CORT during the ACTH challenge). Normality of the residuals from all general linear mixed models was assessed visually using histograms and Q-Q plots. To achieve normality and equal variances, we transformed values for offspring plasma CORT levels (square root) and body mass (natural log). Again, fixed effects included the maternal social environment, offspring sex, and their interactions. In addition, models of offspring growth included a categorical fixed effect of age (in days) to model the increase in weight with age. The models also included all two-way and three-way interactions of age with the maternal social environment and sex to test whether the weight increase with age differed between treatments and sexes. Age was treated as a categorical fixed effect because offspring mass was measured at only three time points (day 0, day 9 and day 19). Models analysing effects on plasma CORT during the ACTH challenge included a fixed effect of sample (pre or post-challenge) to test whether CORT increased in response to the challenge. The models also included all two-way and three-way interactions of sample with the maternal social environment and offspring sex to test whether the response to the challenge differed between treatments and sexes. In addition, the models on plasma CORT included as a covariate the time it took to collect the first sample after the initial disturbance of opening the cage.

Maternal cage was included as a random effect in all models, to control for potential non-independence of mothers from the same cage. In addition, models included a random effect of maternal ID nested within maternal cage, except for the models of primary sex ratio (because parentage was only assigned for hatchlings, not for embryos). Models analysing offspring data collected after the day of hatching also included a random effect of offspring cage. When analysing offspring mortality, models did not converge if both maternal ID and offspring cage were included as random effects, even when increasing iterations to $2*10^9$ and trying a number of different optimizers. Offspring cage was therefore removed from these models because it had a smaller effect than maternal ID within maternal cage (see Supplementary Table 8 in Supplementary Data 3.2). When a random effect of offspring cage was used in the models instead of the random effect of maternal ID, the main effect of sex was no longer significant, suggesting that controlling for maternal ID increased the sensitivity to detect intrinsic sex-differences in mortality. For the analysis of growth and the ACTH challenge, we included the additional random effects of offspring ID nested within offspring cage and offspring ID nested within maternal ID nested within maternal cage.

We always started with the full model and stepwise excluded all non-significant ($p > 0.05$) interactions and main effects, apart from the main factors of interest: the maternal social environment and offspring sex. Interactions were always excluded before the main effects involved in the interaction. We determined the significance of fixed effects using likelihood ratio tests comparing the models with and without the factor of interest. The results of all models are reported in Supplementary Data 3.2.

3.3. Results

3.3.1. Offspring sex ratio and mortality

The maternal social environment did not affect primary sex ratio ($\chi^2_{(1)}$: 0.12, p = 0.73; Figure 3.2), sex ratio at hatching ($\chi^2_{(1)}$: 0.20, p = 0.65; Figure 3.2) or sex ratio at day 23 ($\chi^2_{(1)} < 0.01$, p = 0.99; Figure 3.2). Sex ratios did not differ significantly from parity at any stage in either social environment (all z values < 0.75, all p values > 0.45; Supplementary Table 1 in Supplementary Data 3.2), nor was there any evidence of over or underdispersion in sex ratio at any stage (Supplementary Table 2 in Supplementary Data 3.2). In addition, maternal baseline plasma hormone levels did not predict offspring sex ratio at hatching (effect of maternal baseline androgens: $\chi^2_{(1)}$: 1.53, p = 0.22; effect of maternal baseline CORT: $\chi^2_{(1)}$: 0.36, p = 0.55; Supplementary Figure 3.1).

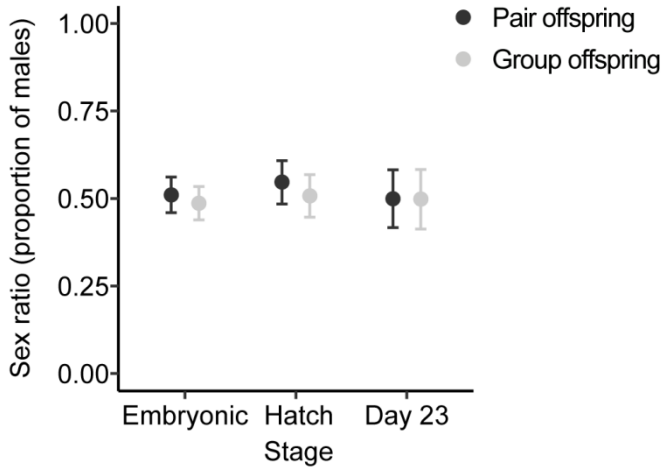


Figure 3.2. Offspring sex ratio at the embryonic stage, at hatching and at day 23. Data shown are the estimated means ± 1 SEM (back-transformed from logit).

The maternal social environment did not have sex-specific effects on offspring mortality (effect of maternal social environment * offspring sex: $\chi^2_{(1)}$: 1.80, p = 0.18; Figure 3.3), nor was there an overall effect of the maternal social environment on mortality ($\chi^2_{(1)}$: 0.20, p = 0.66; Figure 3.3). However, mortality did differ between the sexes: significantly more male offspring than female offspring died before day 23 ($\chi^2_{(1)}$: 4.48, p = 0.03; Figure 3.3). This effect disappeared when the random effect of maternal ID was removed and offspring cage was included, which had a weaker effect but could not be estimated together with

maternal ID in the same model (see methods), suggesting that cage differences in mortality make it difficult to detect sex-differences when effects of maternal ID is not accounted for. Maternal baseline plasma hormone levels did not predict offspring mortality (effect of maternal baseline androgens: $\chi^2_{(1)}$: 1.46, p = 0.23; effect of maternal baseline CORT: $\chi^2_{(1)}$: 0.65, p = 0.42; Supplementary Figure 3.1).

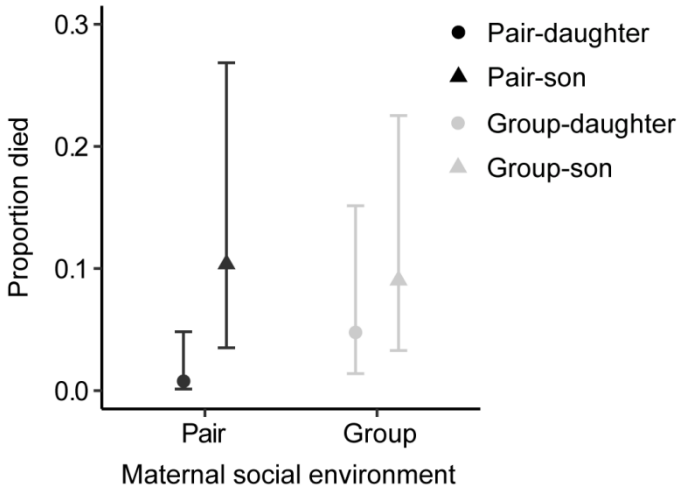


Figure 3.3. The proportion of offspring that died before day 23. Data shown are the estimated means ± 1 SEM (back-transformed from logit).

3.3.2. Egg mass and growth

The maternal social environment had no overall ($\chi^2_{(1)}$: 0.27, p = 0.60; Figure 3.4A) or sex-specific effect on egg weight (effect of maternal social environment * offspring sex: $\chi^2_{(1)}$: 0.02, p = 0.89; Figure 3.4A), nor did egg weight differ between the sexes ($\chi^2_{(1)}$: 0.25, p = 0.62; Figure 3.4A).

All birds increased weight significantly over the course of the experiment (effect of age: $\chi^2_{(2)}$: 1531.30, p < 0.001; Figure 3.4B). The changes in weight with age did not differ between the maternal social environments (effect of maternal social environment * age: $\chi^2_{(2)}$: 0.49, p = 0.78; Figure 3.4B) nor between males and females (effect of offspring sex * age: $\chi^2_{(2)}$: 1.43, p = 0.49; Figure 3.4B) or depending upon the interaction between maternal social environment and offspring sex (effect of maternal social environment * age * offspring sex: $\chi^2_{(2)}$: 4.63, p = 0.10; Figure 3.4B). There was no difference in average offspring mass according to the maternal social environment, offspring sex, or their interaction (the model included the significant effect of age; all $\chi^2_{(1)} < 1.64$, all p values > 0.20).

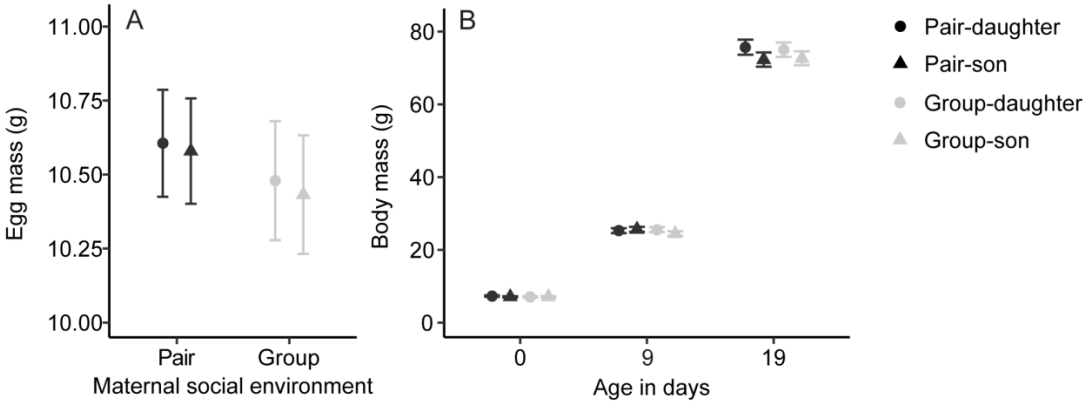


Figure 3.4. A: egg mass. B: offspring growth (back-transformed from natural log). Data shown are the estimated means ± 1 SEM.

3.3.3. Offspring physiology

The maternal social environment had no sex-specific effects on offspring plasma androgen concentrations at hatching (effect of maternal social environment * offspring sex: $\chi^2_{(1)}$: 0.02, p = 0.89; Figure 3.5A). Average androgen concentrations also did not differ between offspring of pair-housed and group-housed mothers ($\chi^2_{(1)}$: 0.45, p = 0.50; Figure 3.5A), nor between males and females ($\chi^2_{(1)}$: 1.92, p = 0.17; Figure 3.5A).

The maternal social environment did not affect the CORT response to an injection with ACTH on post hatch day 20-21 (effect of maternal social environment * sample: $\chi^2_{(1)}$: 0.58, p = 0.45; Fig. 3.5B). Male and female offspring differed in their CORT response (effect of offspring sex * sample: $\chi^2_{(1)}$: 7.11, p < 0.01; Figure 3.5B) but the sex difference in the CORT response was not affected by the maternal social environment (effect of sample * maternal social environment * offspring sex: $\chi^2_{(1)}$: 2.62, p = 0.11). The time between the initial disturbance of opening the cage and the collection of the baseline sample was included as a covariate in all models analysing the effects on ACTH because it significantly affected CORT levels ($\chi^2_{(1)}$: 8.34, p < 0.01). This effect did not differ between offspring from the different maternal social environments ($\chi^2_{(1)}$: 0.19, p = 0.66). Removing the factor "time until the first sample" from these models did not change the significance or interpretation of the main effects. When analysing CORT baseline and response levels separately, male and female offspring did not differ in baseline CORT concentrations ($\chi^2_{(1)}$ = 0.02, p = 0.89; Figure 3.5B), but males had significantly higher CORT concentrations after the ACTH injection ($\chi^2_{(1)}$: 16.33, p < 0.001; Figure 3.5B). CORT concentrations increased significantly in both sexes after the ACTH injection (males: $\chi^2_{(1)}$: 146.79, p < 0.001; females: $\chi^2_{(1)}$: 86.44, p < 0.001; Figure 3.5B).

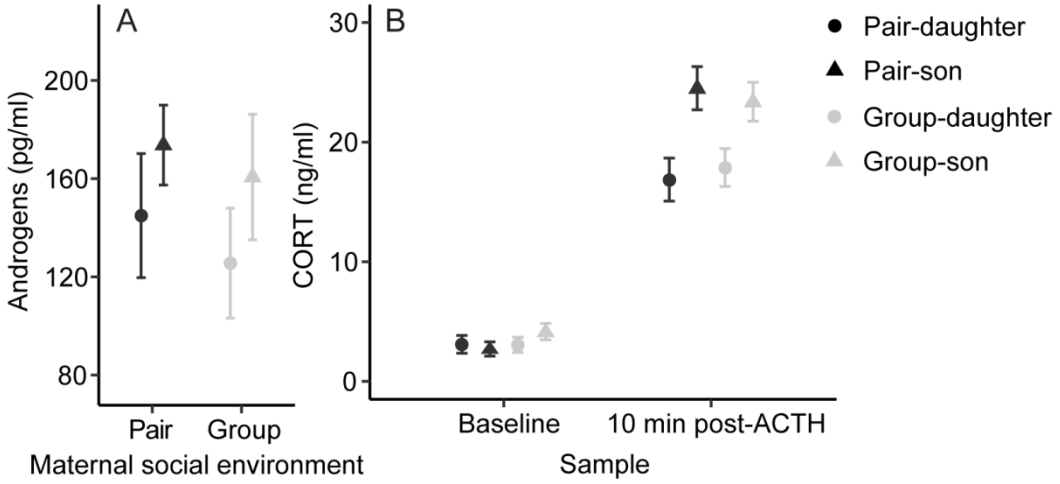


Figure 3.5. A: offspring plasma androgen concentrations (pg/ml) at hatching. B: offspring plasma CORT concentrations (ng/ml) before and 10 minutes after the ACTH injection (back-transformed from square root). Data shown are the estimated means ± 1 SEM.

3.4. Discussion

The social environment a female is exposed to during reproduction has been reported to induce variation in offspring sex-ratio, mortality, development, and endocrine physiology in a number of vertebrate species (Dantzer et al., 2013; Guibert et al., 2010; Kaiser and Sachser, 2009, 2005; Michler et al., 2013; Minias et al., 2014; von Engelhardt et al., 2015). Maternal hormones are candidate signals involved in such transgenerational effects (Dantzer et al., 2013; Guibert et al., 2010; Hayward and Wingfield, 2004; Henriksen et al., 2013), and they are thought to represent important proximate mechanisms in adaptive sex allocation (Navara, 2013a, 2013b), also by affecting secondary offspring sex ratios (Love et al., 2005; Rutkowska et al., 2007; Rutkowska and Cichoń, 2006).

We did not find evidence that the maternal social environment (pair versus group housing) affects offspring sex ratio in Japanese quail, even though pair-housed females had increased circulating androgen levels and a non-significant trend of higher cortisol levels compared to group-housed females, as reported in our previous study (Langen et al., 2017). We had predicted that pair-housed females would produce a female-biased offspring sex-ratio because increased androgen and CORT levels were associated with a female-biased sex-ratio in other studies on Japanese quail (Correa et al., 2011, Pike and Petrie, 2006). Offspring from pair-housed mothers and offspring from group-housed mothers also did not differ in growth, mortality, circulating androgen levels or circulating CORT levels. Moreover, maternal circulating levels of androgens and CORT did not correlate with offspring sex ratio and mortality.

Our results contradict the general pattern in avian species which suggests that higher maternal androgens lead to a male-biased offspring sex ratio (Goerlich et al., 2009; Navara, 2013a, 2013b), but we corroborate earlier findings in Japanese quail showing no such relationship (Pike and Petrie, 2006). However, in Japanese quail, higher maternal androgens have also been linked to female-biased sex ratios (Correa et al., 2011), indicating

that the effect of maternal androgens on offspring sex ratio is still unclear. Our results also do not confirm earlier reports that higher maternal CORT levels are linked to a female-biased offspring sex ratio in avian species (Navara, 2013a, 2013b), including Japanese quail (Pike and Petrie, 2006). Studies investigating the relationship between maternal plasma hormone levels and offspring sex ratio differ substantially regarding methods of hormone manipulation or quantification which might explain differing results between studies. For example, Correa et al. (2011) found the temporary peak in circulating androgen levels following mating to be correlated with a female-biased sex ratio. On the other hand, Pike and Petrie (2006), who found no relationship between offspring sex ratio and androgens, analysed faecal androgen metabolite concentrations, which do not reflect short-term fluctuations but an integrated measure of androgen concentrations over several hours. They also found no effect of treating females with androgen implants, which likely affected circulating androgen levels over a longer time period. In addition, multiple steroid hormones are thought to be involved in sex ratio adjustment, and their effects may interact (Navara, 2013a). In the present study, the opposing effects of higher maternal androgens and higher CORT on offspring sex ratio may have cancelled each other out, explaining why the offspring sex ratio of pair-housed mothers did not differ from parity nor from that of group-housed females. Moreover, the elevation in maternal plasma androgen and CORT levels in pair-housed females may not have been large enough to induce a shift in offspring sex ratio. Finally, it has been suggested that effects on primary sex ratios may be largely due to variation in levels of progesterone during meiosis, which is the main follicular steroid produced during this phase (Correa et al. 2005) but was not measured in our study.

The lack of an effect of the maternal social environment on offspring growth, mortality or physiology might partly be explained by the fact that the maternal social environment did not induce differences in yolk androgens, as shown in our previous study (Langen et al., 2017), or in egg mass, as shown here. Yolk hormones are considered a key mechanism in transferring the effects of the maternal social environment to offspring (Gil, 2008; Rutkowska and Cichoń, 2006; von Engelhardt et al., 2006; von Engelhardt and Groothuis, 2011), and differences in the maternal social environment and physiology alone may not suffice to induce changes in the prenatal environment. The fact that we found no effects on egg mass can also explain why offspring growth and mortality did not differ, egg size being another important mediator of maternal effects (Cunningham & Russell 2000; Hadfield et al. 2013; Krist, 2011; Pick et al. 2016; Williams, 1994). We also found no sex differences in egg mass, confirming previous suggestions that there is little evidence overall for sexual size dimorphism in eggs across avian species (Rutkowska et al. 2014).

We did find a difference in the physiological stress response (increase in CORT) after an ACTH injection between male and female offspring, irrespective of the maternal social environment. While baseline CORT concentrations did not differ between males and females, males showed a higher CORT response, suggesting increased sensitivity of their HPA axis. This is in line with many studies in birds, including Japanese quail, that report a stronger stress response in males compared to females (Astheimer et al., 1994; Goerlich et al., 2012; Hayward et al., 2006; Hazard et al., 2008; Krause et al., 2015; Madison et al.,

2008; Romero et al., 2006; Schmeling and Nockels, 1978). Other studies, however, report no differences (Dufty Jr. and Belthoff, 1997; Hazard et al., 2008; Satterlee and Johnson, 1988; Sockman and Schwabl, 2001). In addition to having a higher stress response, significantly more male offspring died before day 23 than female offspring. Similar patterns in juvenile mortality are reported in a large number of species (reviewed by Clutton-Brock, 1991), suggesting that males are more vulnerable to environmental challenges. However, it is still unclear what the underlying mechanisms are (Jones et al., 2009). Interestingly the sex-difference in mortality was only detected when controlling for maternal ID, not when controlling for offspring cage. This made it harder to estimate intrinsic sex-differences in mortality because offspring from the same mother were allocated to different offspring cages so that the sex-differences in mortality could be attributed to cage effects. While perhaps not surprising, this also suggests that it is important to consider how the genetic effects, maternal environmental effects and the posthatching environment may interact in affecting sex-specific mortality.

Overall, contradictory findings regarding the effects of maternal physiology and maternal social environment on offspring sex ratio and phenotypes indicate that the mechanisms underlying such maternal effects are still insufficiently understood. An important factor explaining differences between studies, including our own and previous research, might be the timing of manipulations and measurements of the social environment and the endocrine system. Effects of the maternal environment and physiology on developing follicles and offspring may occur only during critical windows (Okuliarova et al., 2017). For example, for sex ratio adjustment, an influence of maternal steroids on the segregation of the sex chromosomes during the first meiotic division has been proposed (Correa et al., 2005; Goerlich-Jansson et al., 2013; Navara, 2013a, 2013b; Pinson et al., 2011; Rutkowska and Badyaev, 2008). Also, circulating hormone levels differ between life stages and seasons and can change significantly during a single day, even within minutes, in response to the environment, such as social stimuli (Adkins-Regan, 2005; Creel et al., 2013; Hazard et al., 2005; Oliveira, 2004; Ottinger et al., 2001). A single measurement of physiological status does not take such fluctuations into account and might reduce the chance of detecting maternal effects. We may have also missed important effects by not measuring maternal hormones during the time window during which genetic sex determination takes place (meiosis I) and by only measuring maternal androgens and corticosterone, not other steroids such as progesterone (Correa et al., 2005).

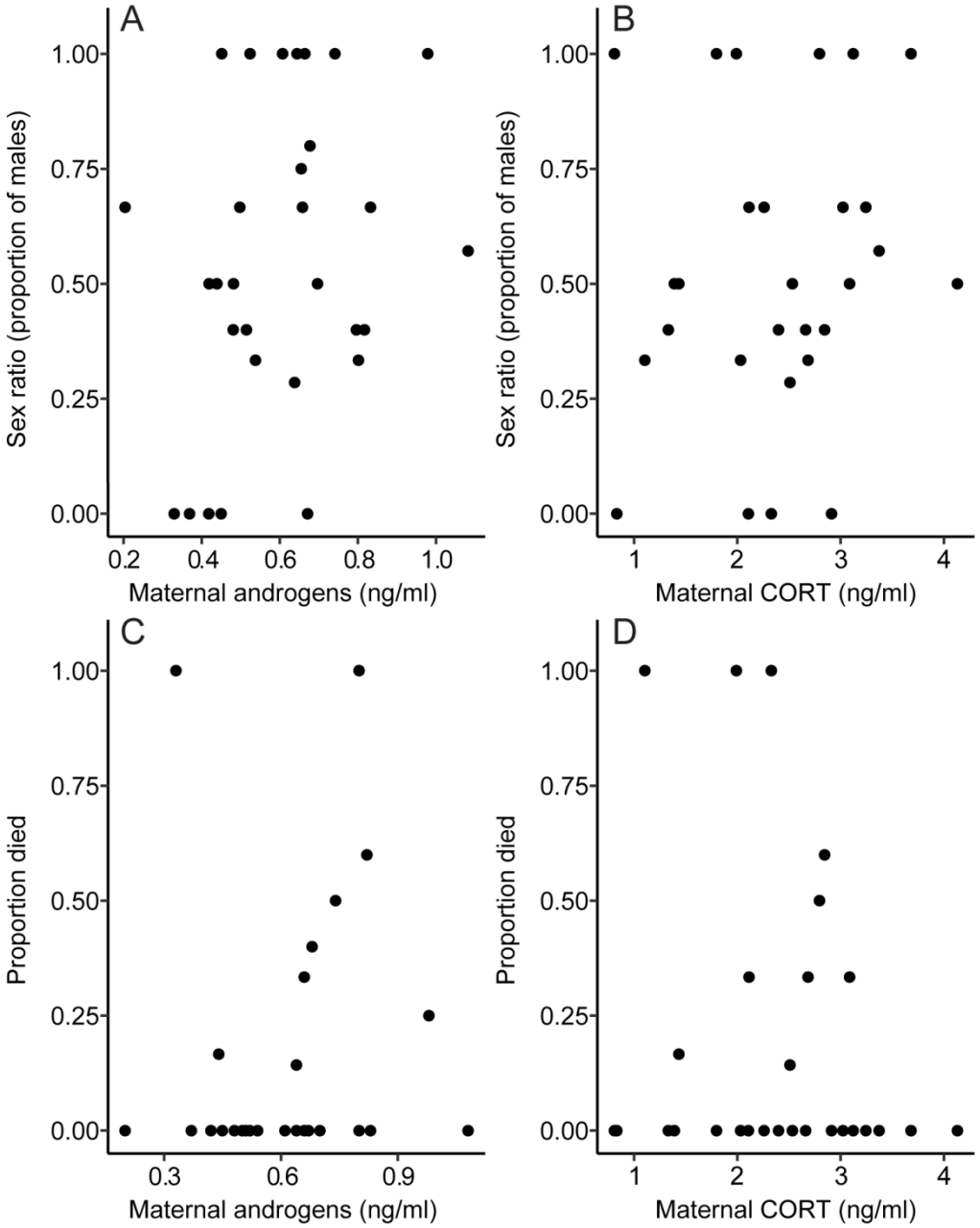
Finally, differences between the social stimuli investigated may explain the contradictory results between studies. Social factors such as maternal social instability (Guibert et al., 2010; Kaiser and Sachser, 2009), social density (Dantzer et al., 2013; Minias et al., 2014; von Engelhardt et al., 2015), mate attractiveness (Kölliker et al., 1999; Korsten et al., 2006; Rutstein et al., 2005; Sheldon et al., 1999; Svensson and Nilsson, 1996), pair bonding (Hirschenhauser, 2012; Le Bot et al., 2014; Schweitzer et al., 2014), and social status (Dloniak et al., 2006) are likely to differ in their functional significance and therefore also in their effects on offspring phenotypes and sex ratio. To gain a better understanding of the underlying mechanisms and the function of maternal effects of the social

environment, it is therefore necessary to establish which social stimuli are most important for offspring, and at which time maternal effects manifest in relation to the prenatal and postnatal developmental stages.

3.5. Acknowledgements

We thank Aline Bertin and her colleagues at the INRA in Nouzilly, France for their helpful advice and for providing us with our first generation of Japanese quail. We also thank Irene de la Casa, Kathrin Engel, Sarah Golüke, Judith Hendriks, Elke Hippauf and Susanne Kirchhoff for their help in the lab and with the experimental procedures, the animal caretakers for looking after the birds, and Suzanne von Engelhardt for English editing of the manuscript. We gratefully acknowledge two anonymous referees for providing constructive comments on the manuscript. This research was funded by grant support from the Volkswagen Foundation (Az 86 005, acquired by VCGJ). The funding source had no role in study design, data collection or analysis, preparation of the manuscript, or the decision to submit the manuscript for publication.

Supplementary material



Supplementary Figure 3.1. Relationship between maternal hormones and offspring sex ratio at hatching and mortality. A: maternal androgens and offspring sex ratio at hatching, B: maternal CORT and offspring sex ratio at hatching, C: maternal androgens and offspring mortality, D: maternal CORT and offspring mortality.

Supplementary Data 3.1. Raw dataset; available at https://doi.org/10.1016/j.ygcen.2018.04.015

Supplementary Data 3.2. Supplementary tables, including summaries of all model outputs; available at https://doi.org/10.1016/j.ygcen.2018.04.015

Chapter 4

Effects of the maternal and current social environment on female body mass and reproductive traits in Japanese quail (*Coturnix japonica*)

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Abstract

The social environment can affect the phenotype of breeding females and their offspring. Using Japanese quail (*Coturnix japonica*), we studied maternal effects of the social environment on female offspring housed under matched and mismatched conditions with respect to group size (pairs of two vs. groups of four birds). We measured F1 growth, reproduction and plasma levels of androgens and corticosterone and found a match-mismatch effect on F1 body mass but not on other traits. F1 group housing led to faster growth, but had an additional positive effect on weight only in daughters of pair-housed females. At the time of egg collection for the F2 generation, F1 group females were heavier, irrespective of the P0 treatment. This resulted in a maternal effect on F2 offspring with females in groups laying heavier eggs with a higher hatching success and producing heavier offspring. Neither the P0 nor the F1 social environment affected F1 plasma androgen or corticosterone levels, suggesting effects on growth and reproduction are not necessarily reflected in hormonal differences. The effects of social environment on females and their offspring differed between the P0 (reported previously) and F1, most likely because of different sex ratios in the social treatment groups. The fitness consequences of the observed maternal effect and the role of different social influences, such as group size and sex ratio on offspring, require further study to understand their adaptive significance and physiological mechanisms.

4.1. Introduction

Effects of the maternal social environment on female physiology, reproduction and offspring phenotype have been described in various species, including birds and mammals (Groothuis et al., 2005; Guibert et al., 2010; Kaiser and Sachser, 2009, 2005). Maternal effects can act as mechanisms of adaptive transgenerational plasticity to optimally prepare offspring phenotype for their future environment. This can be tested by studying the consequences for offspring experiencing an environment that matches or mismatches the maternal environment (Burgess and Marshall, 2014; Marshall and Uller, 2007; Uller et al., 2013). This study investigates the transgenerational effects of maternal social group size on offspring housed under either matched or mismatched social conditions in an avian species, the Japanese quail (*Coturnix japonica*).

Behaviour, physiology and reproduction can be affected by the social environment, such as density, group size, social rank, mate attractiveness or adult/operational sex ratio (Alonso-Alvarez et al., 2012; Asghar Saki et al., 2012; Benyi et al., 2006; Both, 1998; Both et al., 2000; Clutton-Brock and Huchard, 2013; Cunningham and Russell, 2000; Dewsbury, 1982; Ellis, 1995; Fowler, 1981; Rodenhouse et al., 2003; Schubert et al., 2007; Sillett et al., 2004; Stockley and Bro-Jørgensen, 2011; Székely et al., 2014; Uller et al., 2005). Effects of the social environment on female endocrine physiology and body mass (Bonenfant et al., 2009; DeVries et al., 2003; Eisenegger et al., 2011) provide proximate mechanisms through which reproduction and offspring can be affected. In birds, increasing group size, for example, is thought to exacerbate intraspecific competition which can affect body mass (Asghar Saki et al., 2012; Keeling et al., 2003; Onbaşılar and Aksoy, 2005) and circulating levels of steroid hormones such as corticosterone and androgens (Cantarero et al., 2015; Cunningham et al., 1987; Koelkebeck and Cain, 1984; Langmore et al., 2002; Mazuc et al., 2003; Onbaşılar and Aksoy, 2005; Raouf et al., 2006; Smith et al., 2005). In Japanese quail, frequent changes in the group composition of breeding females are thought to reflect increased social densities and lead to elevated plasma corticosterone concentrations (Guibert et al., 2010). In contrast, Japanese quail females housed in pairs had higher circulating androgen levels and tended to have higher circulating corticosterone levels than group-housed females (Langen et al., 2017). Such effects of the social environment on female physiology and body mass and condition may affect their ability to invest in reproduction, resulting in changes in the quality or quantity of eggs produced or the quality or quantity of the offspring (Christians, 2002; Drent and Daan, 1980; Lim et al., 2014; Ronget et al., 2018; Sockman et al., 2006). Studies have reported both positive and negative correlations between measures of reproduction and circulating androgens (positive: Cain and Ketterson, 2012; Langmore et al., 2002; Sandell, 2007; negative: de Jong et al., 2016; López-Rull and Gil, 2009; Rutkowska et al., 2005; Rutkowska and Cichoń, 2006; Veiga and Polo, 2008) and glucocorticoids (positive: Bonier et al., 2009b; Burtka et al., 2016; Ouyang et al., 2013, 2011; negative: Angelier et al., 2010; Bonier et al., 2009b; Ouyang et al., 2013, 2011; Silverin, 1986; Vitousek et al., 2014).

Effects of the social environment on female physiology and reproductive investment can lead to effects on offspring development and fitness. Kaiser et al. (2003)

found in guinea pigs (*Cavia aperea*), for instance, that maternal social instability resulted in decreased maternal plasma androgen concentrations and affected offspring behaviour and physiology. Daughters of unstable mothers were masculinized in their behaviour and had increased plasma androgen concentrations during adulthood, whereas sons were infantilized. In American red squirrels (*Tamiasciurus hudsonicus*), higher maternal social densities increased maternal corticosterone and offspring growth rates (Dantzer et al., 2013). In Japanese quail (*Coturnix japonica*), maternal social instability reduced offspring growth during the first weeks of life (Guibert et al., 2010). Maternal effects on growth and physiology may influence offsprings' future reproduction since an individual's reproductive performance often depends on its body condition and/or endocrine status (Burtka et al., 2016; Correa et al., 2011; de Jong et al., 2016; Devries et al., 2008; Festa-Bianchet et al., 1998; López-Rull and Gil, 2009; Milenkaya et al., 2015; Ouyang et al., 2013, 2011; Rutkowska et al., 2005; Veiga and Polo, 2008). However, the adaptive significance of maternal effects induced by social stimuli is still insufficiently understood.

In the present study we investigate the potential interactive effects of the maternal and offspring social environment. Females of the parental (P0) generation were housed in pairs (one female and one male) or in groups (three females and one male) and allowed to reproduce (Langen et al. 2017). The females of the offspring (F1) generation were similarly housed in either pairs of two females or groups of four females, with daughters from the two maternal conditions evenly allocated to the two F1 social conditions. This allowed us to investigate the effects of the P0 social environment, the F1 female's own social environment, and their interaction on physiology (growth and circulating levels of corticosterone and androgens) and reproduction (egg production, egg mass, fertilization rates, hatching success, and offspring mass). We assessed the sensitivity of the F1 female's hypothalamic-pituitary-adrenal (HPA) axis using a standardized restraint stress challenge (Wingfield et al., 1995) and assessed the responsiveness of the hypothalamic-pituitary-gonadal (HPG) axis using a gonadotrophin-releasing hormone (GnRH) challenge (Jawor et al., 2006; Peluc et al., 2012). This enabled us to investigate whether effects on reproductive performance reflect physiological changes during reproduction (e.g., Angelier et al., 2010; Bonier et al. 2009b, Burtka et al., 2016, Cunningham et al., 1987, Ouyang et al., 2011, 2013).

Adaptive effects of the maternal social environment should prepare their offspring for the social environment anticipated by the mother's social experience. We therefore expected F1 female offspring to show better growth and reproduction under social conditions matching the maternal environment compared to the female offspring housed under mismatched social conditions.

4.2. Materials and methods

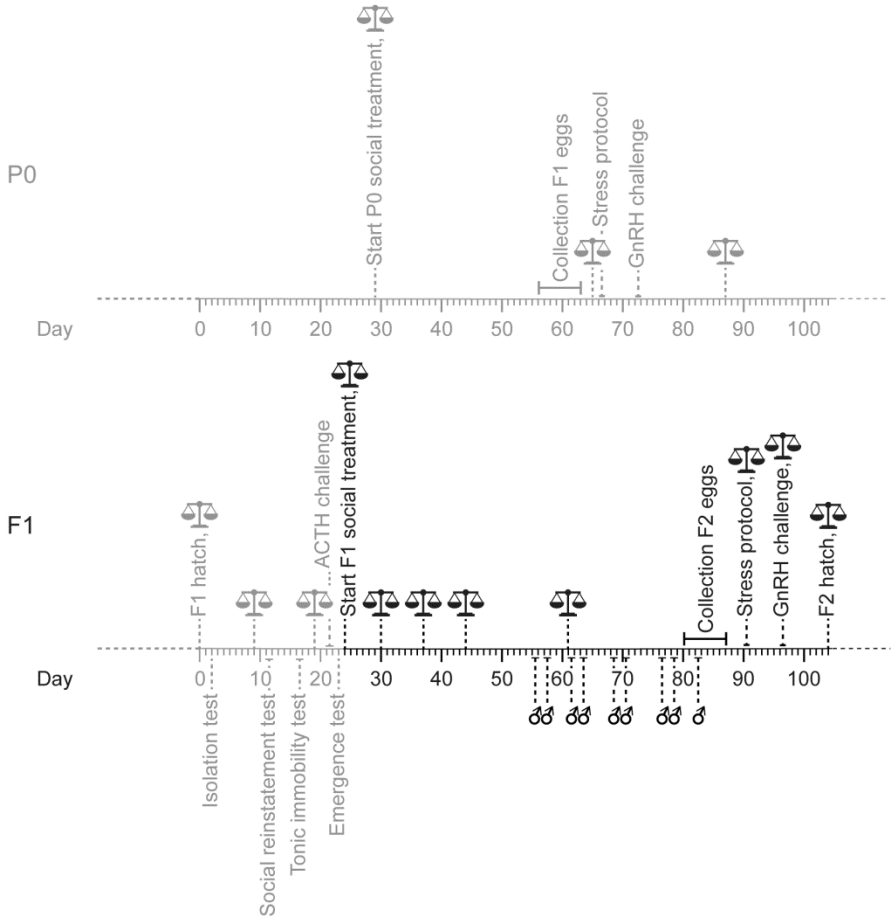

Figure 4.1. Timeline of experimental procedures. Measurements in grey are not presented here, but some of these are published elsewhere (see Langen et al., 2017, 2018 for more information). Scale symbols indicate when animals were weighed. ♂ indicates when females and males were brought together for mating.

4.2.1. Ethics statement

All experimental procedures were approved by the North Rhine-Westphalia State Agency for Nature, Environment and Consumer Protection (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen), Recklinghausen, Germany (licence number 84-02.04.2013-A127). Animal facilities were approved for keeping and breeding Japanese quail for research purposes by the local government authority responsible for health, veterinary and food monitoring (Gesundheits-, Veterinär- und Lebensmittelüberwachungsamt Bielefeld, Germany).

4.2.2. Origin of the parental generation

The eggs from which the parental generation hatched were provided by the INRA in Nouzilly, France (Experimental unit 1295 (UE PEAT) and UMR 85, Physiologie de la Reproduction et des Comportements, INRA-CNRS-IFCE-Université de Tours, Val de Loire

Center, Nouzilly, France). The eggs were laid by females from a non-selected control line, bred next to quail lines selected for low or high social reinstatement (Mills and Faure, 1991).

4.2.3. Social environments

Females were housed under two different social conditions shortly before sexual maturity: P0 females were housed in pairs (one female with one male) or in groups (three females with one male) and F1 females were housed in pairs (two females, one offspring from each of the P0 treatments) or in groups (four females, two offspring from each of the P0 treatment). The birds were placed in the experimental social conditions at the age of 29 days in the P0 generation (Langen et al., 2017) and 24 days in the F1 generation, about two weeks before the onset of egg laying. At that time the birds were still unfamiliar with each other. Siblings and half-siblings (in the P0) or cousins (in the F1) were never housed in the same cage. F1 males (n=15, all offspring from the P0 pair treatment) were housed in single cages and only encountered females for mating. Males were not housed with females in the F1 generation to avoid injury to the females which could result from high copulation frequency when housed in pairs (see Langen et al., 2017).

In the P0 generation, 17 pair-housed females and 20 group-housed females produced F1 offspring (Langen et al., 2017). Thirteen of the pair-housed females and 13 of the group-housed females produced the 53 daughters used in the current experiment. These F1 females were evenly allocated to 16 pairs and 7 groups, balanced with respect to maternal treatment. We thus created four different treatments in the F1 generation, representing all combinations of the P0 and F1 social conditions: daughters from pair-housed mothers housed in pairs ($P_{P0}P_{F1}$, n=16), daughters from pair-housed mothers housed in groups ($P_{P0}G_{F1}$, n=11), daughters from group-housed mothers housed in groups ($G_{P0}G_{F1}$, n=13), and daughters from group-housed mothers housed in pairs ($G_{P0}P_{F1}$, n=13). Three pair cages and three group cages contained females that were not used for the experimental tests, but served as cage mates for the experimental birds (see also Appendix B). These seven females were the offspring of P0 birds that had been excluded from the experiments due to aggression (for more information, see Langen et al., 2017). For details on sample sizes, see Table 4.1.

Due to aggression, we had to separate 11 pairs and 4 groups in the F1 generation over the course of the experiment. Of the 11, 10 pairs were separated using a wire mesh so that visual, acoustic and limited tactile interaction was still possible, and they were kept in our experiment. One pair was completely separated and removed from the experiment because one of the females had wounds that were unlikely to heal within a few days, constituting a humane endpoint. The four groups had to be fully separated because it was not possible to use a wire mesh in their cage to keep them apart and allow visual, acoustic and tactile interaction. The study included only data from before the separation of the one pair and the four groups. Therefore each measurement had a different sample size (for exact sample sizes, see Tables 4.1-4.2 and Figures 4.2-4.4). For more details on when the birds were separated, see Supplementary Data 4.1.

Table 4.1. Experimental groups and sample sizes (number of females in the two P0 social treatments and in the four combinations of F1 social treatments): ahoused in 16 F1 pair cages; bhoused in 7 F1 group cages; c7 P0 pair-housed mothers contribute to both F1 pairs and F1 groups; d6 P0 group-housed mothers contribute to both F1 pairs and F1 groups.

Maternal social environment	P0 females	Own social environment	F1 Females (P0 mothers)
P_{P0}	13	P_{F1}[a]	16 (11[c])
		G_{F1}[b]	11 (9[c])
G_{P0}	13	P_{F1}[a]	13 (10[d])
		G_{F1}[b]	13 (9[d])

4.2.4. Animal husbandry

All birds were housed in two adjacent rooms in the P0 generation (Langen et al., 2017) and three adjacent rooms in the F1 generation (two rooms for the females and one room for the males). All rooms had artificial lighting and ambient temperature, with a minimum temperature of 20˚C. Main lights were set to a 14:10h light-dark cycle (lights on at 5 am), except for the first day and night after hatching when lights remained on for 24 hours. Cages never faced each other to prevent visual contact between birds from different cages, but acoustic and olfactory communication was possible.

In the P0 generation, pairs were kept in cages measuring 75 x 80 x 40 cm, groups in cages measuring 150 x 80 x 40 cm. The adult F1 females were all kept in cages measuring 150 x 80 x 40 cm, irrespective of their social conditions. Males were housed in cages measuring 75 x 80 x 40 cm. Birds were kept on wood shavings, and all cages contained a sand bath and one shelter hut per bird. Food (GoldDott Hennenmehl, Derby Spezialfutter GmbH, Münster, Germany) and water was provided ad libitum. On a weekly basis, the standard diet was supplemented with mealworms and shell grit.

Females were weighed before they were housed in their adult social condition on day 24, and on days 30, 37, 44, 61, 90 and 97.

4.2.5. Mating

Females of the F1 generation were housed in single-sex groups but had temporary access to males for mating (see Figure 4.1). In each mating session, males and females were together for 20 minutes. Fifteen males, all sons of pair-housed females, were used in total, and females were always paired with the same unrelated male (not sharing the same grandparents). Each male was paired with four different females, one from each combination of the P0 and F1 social conditions, except for one male who was only paired to $P_{P0}P_{F1}$ females. On days 55-56 males were introduced into the home cages of the females and allowed to mate for 20 minutes. Since the male was unable to copulate with the two or four females in a cage within such a short time period, we subsequently paired him with one female at a time in the next mating sessions. Each female was paired twice a week and each male was paired with the same two females per day, but in alternating order.

Furthermore, we started the mating sessions with a different male and female every day so that the pairing order was randomised for males as well as females. On days 57-58 and 61-64, females and males were paired in a neutral mating cage between 08:00 and 17:00. Thereafter, on days 68-69, 70-71, 76-77, 78-79 and 82-83 females were introduced to the male's home cages between 10:00-12:30.

4.2.6. Egg collection for the F2 generation, incubation and hatching

Eggs for the F2 generation were collected on days 80 - 87. All eggs were stored at 16°C until the end of the collection period (storage time ranging between 1 - 7 days) when incubation started. All eggs were incubated at the same time in a HEKA-Euro-Lux II incubator (HEKA-Brutgeräte, Rietberg, Germany). Incubation was done in complete darkness to avoid the effects of light on development and because it more likely reflected the situation during natural incubation (Archer and Mench, 2014). From incubation day 1 to day 14, the temperature was set at 37.8°C, humidity at 55%, and the eggs were turned every 2 hours. Eggs were candled after 9 days of incubation to identify embryonic development. Non-fertilized eggs were removed (see Table 5 for number of eggs and fertilization). From day 15 onwards, the incubation temperature was set at 37.5°C, the humidity at 75%, and the eggs were no longer turned. After 15 days of incubation, eggs were placed in separate compartments (5.5 x 5.5 x 5 cm) on hatching trays. The individual compartments allowed us to identify which chick hatched from which egg. The compartment walls were made of transparent Plexiglas and the bottom of each hatching tray was made of mesh wire, allowing air flow and olfactory and acoustic communication between the chicks.

All eggs hatched after 17 ± 1 days of incubation. Hatchlings were removed from the incubator once their feathers had dried (ca. 2 hours after hatching) and weighed to the nearest 0.1 g. A blood sample (max. 50 µl or about 0.5% of body weight; <0.8% does not appear to have long-term effects on adult or developing birds; Sheldon et al. 2008) was taken for assignment of parentage. Blood sampling was done by piercing the jugular vein with a sterile 27-gauge needle and collecting the blood in heparinized capillaries (BRAND GMBH + CO KG, Wertheim, Germany).

4.2.7. Parentage assignment

F2 hatchling blood was centrifuged for 10 minutes at 2000 x g. Blood cells were diluted 1:2 with phosphate buffer saline (10 mM PBS+6 mM EDTA, pH 7.4) and stored at -20°C. We used a small sample of blood from the stress protocol or GnRH challenge from the adult F1 females. Genomic DNA was obtained by a phenol/chloroform or Chelex extraction (Walsh et al., 1991). Parentage was manually assigned after genotyping all parents and offspring at 22 microsatellite loci using fluorescently labelled primers, as described previously (Langen et al., 2017).

4.2.8. Stress protocol and GnRH challenge

The stress protocol and the GnRH challenge were performed after collecting the F2 generation eggs to exclude effects on reproduction. The stress protocol took place on days 90 - 91. All birds were tested between 09:20 am - 12:30 pm and corticosterone levels did not change significantly during that period ($\chi^2_{(1)}$ = 0.30, p = 0.58). After catching the birds from their home cages, a blood sample was taken within 3 minutes to determine baseline plasma corticosterone concentrations by puncturing the ulnar vein with a sterile needle and collecting 200 - 300 µl blood in heparinised capillaries (BRAND GMBH + CO KG, Wertheim, Germany). After taking the baseline sample, the birds were restrained for 10 minutes by placing them in a cotton bag (Ecotone, 25 x 30 cm). A second blood sample was taken after the 10-minute restraint period to determine the female's corticosterone response (in total, 2 x 200 - 300 µl blood was collected on the days of the stress protocol and the GnRH challenge, or about 0.18% - 0.28% of body weight at those ages; <0.8% does not appear to have long-term effects on adult or developing birds; Sheldon et al., 2008).

The GnRH challenge took place on days 96 - 97 while all females were laying eggs and thus assumed to be responsive to GnRH (Jawor et al., 2006; Peluc et al., 2012). All birds were tested between 09:25 am - 12:30 pm. As in the stress protocol, birds were caught, and a blood sample was taken from the ulnar vein within 3 minutes to determine baseline plasma androgen concentrations. After the baseline sample was taken, the females were injected in the pectoral muscle with 5 µg chicken GnRH-I (H-3106, APC number 54-8-23, CAS No: 47922-48-5, Bachem, Bubendorf, Switzerland, formerly also sold as Sigma-L0637) dissolved in 50 µl PBS, and returned to their home cages. Thirty minutes post injection, the birds were caught again, and a second blood sample was taken to determine the female's plasma androgen concentration in response to GnRH.

4.2.9. Hormone analysis

Blood samples from the stress protocol and the GnRH challenge were kept on ice for a maximum of two hours after sampling and then centrifuged for 10 minutes at 2000 x g. Following centrifugation, plasma was collected and frozen at -20°C.

Plasma corticosterone concentrations were determined using a commercial corticosterone radioimmunoassay kit (MP Biomedicals, Orangeburg, USA, cat. no. 07-102102). Cross-reactivity of the kit antibody was 0.34% for desoxycorticosterone, 0.1% for testosterone, and less than 0.1% for all other steroids tested (as reported by the manufacturer). Samples were measured together with quail plasma samples from other experiments and were distributed over 10 assays with an average intra-assay coefficient of variation (CV) of 4.78%, and an inter-assay CV of 7.13% (based on a chicken plasma pool and 2 kit controls measured in duplicate in each assay). Across assays, samples were balanced for treatment.

Plasma androgen concentrations were determined using a commercial T enzyme immunoassay kit (Demeditec Diagnostics GmbH, Kiel, Germany, cat. no. DES6622). Cross-reactivity of the kit antibody was 23.3% for 5α-dihydrotestosterone, 1.6% for

androstenedione, and less than 0.1% for other tested steroids (as reported by the manufacturer). Samples were measured together with quail plasma samples from other experiments and were distributed over 9 assays with an average intra-assay coefficient of variation (CV) of 4.38% (based on all plasma samples measured in duplicate), and an inter-assay CV of 13.82% (based on 2 control plasma pools measured in each of the 9 assays). Across assays, samples were balanced for treatment.

4.2.10. Statistical analysis

Data were analysed using R 3.4.3 (R Core Team, 2017), package lme4 (Bates et al., 2015). General linear mixed models were fitted for growth, mass around egg collection, egg mass, F2 mass at hatching and plasma hormones. Analysis of egg laying, fertilization and hatching success was done using generalised linear mixed models with a binomial error distribution and logit link function. To control for the non-independence of F1 offspring from the same P0 mother, we always included P0 mother as a random effect. We also included a random effect of F1 female nested within P0 mother for repeated measurements from the same F1 female (growth, egg laying rate, fertilization and hatching success and plasma hormones).

All models included P0 social environment, F1 social environment and their interaction as fixed effects. Models analysing plasma hormones included an additional fixed effect of sample, and its two-way and three-way interaction with the P0 and F1 social environment. Models analysing growth included a linear, quadratic and cubic effect of age in days ($day + day^2 + day^3$) to model the non-linear relationship between age and mass. In addition, the two-way and three-way interactions between ($day + day^2 + day^3$) and the P0 and F1 social environment were included. The female's age in days was centered around the mean age within our dataset by subtracting 45 from each age. The intercept and main effects of the models therefore represent the estimated weight at day 45.

We tested whether effects on F1 female mass could explain differences in F2 egg mass by including F1 females mass at day 90 (close to the period of egg collection) as a covariate in the model. Similarly, we included egg mass as a covariate in models testing effects on F2 mass at hatching.

We started out with the full models, including all interactions, and then stepwise excluded all non-significant predictors or interactions ($p > 0.05$), except for the main parameters of interest, i.e. social treatment, age in days ($day + day^2 + day^3$; for growth) and sample number (for hormonal responses). Interactions were always excluded before the main effects involved in the interaction. We determined the significance of fixed effects using likelihood ratio tests, comparing the models with and without the parameter of interest. Distributions of model residuals were visually assessed for normality and homoscedasticity using histograms and Q-Q plots. Plasma corticosterone concentrations were log10-transformed to achieve normality. The results of all models are reported in Supplementary Data 4.1, and the dataset used for analyses is reported in Supplementary Data 4.2.

4.3. Results

4.3.1. Growth, mass around egg collection, egg mass and offspring mass

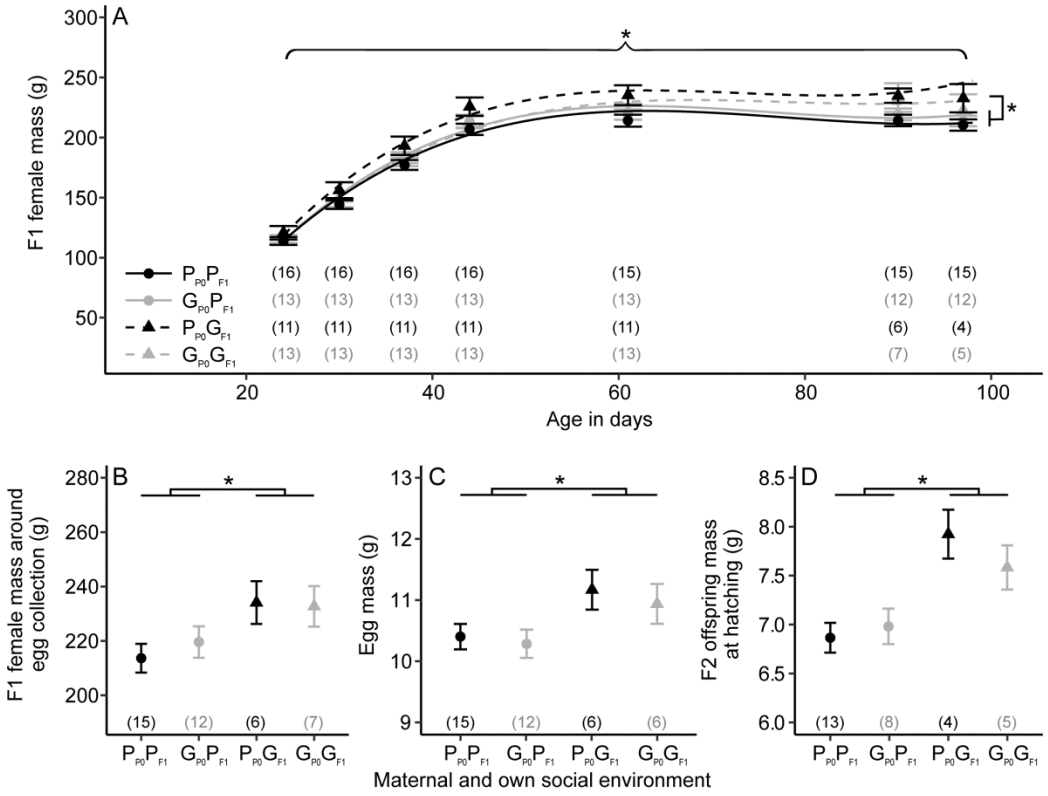

Figure 4.2. A: Female growth. Females housed in groups in the F1 (triangles and dashed lines) grew faster than F1 females housed in pairs (circles and solid lines). In addition, F1 group housing had a positive effect on weight, but only in daughters of pair-housed females, not of group-housed females. B: average female mass around egg collection (day 90). Females housed in groups were significantly heavier than females housed in pairs. There was no effect of the maternal social environment or its interaction with the female's own social environment. C: egg mass. Females housed in groups laid significantly heavier eggs than females housed in pairs. There was no effect of the maternal social environment or its interaction with the female's own social environment. D: F2 offspring mass. Females housed in groups had significantly heavier F2 offspring than females housed in pairs. There was no effect of the maternal social environment or its interaction with the female's own social environment. Data shown in figure 4.2A are the raw means ± 1 SEM, with lines indicating model predictions. Data shown in figure 4.2B, C and D are the estimated means ± 1 SEM. Numbers between brackets indicate the number of F1 females included (for number of F2 offspring, see Table 4.2).

Females housed in groups grew faster than females housed in pairs (own social environment*(day + day² + day³): $\chi^2_{(3)} = 21.94$, $p < 0.001$; Figure 4.2A). In addition, there was a significant effect of the interaction between the P0 maternal social environment and F1 own social environment on female mass ($\chi^2_{(1)} = 4.14$, $p = 0.04$). The dataset was split according to maternal social environment for further post-hoc testing. This analysis revealed that F1 group housing had a positive effect on growth in daughters of pair-housed

mothers and no effect on growth in daughters of group-housed mothers (see Supplementary Data 4.1 for more details). At day 90, close to the period of egg collection for the F2 generation, females housed in groups were significantly heavier than females housed in pairs ($\chi^2_{(1)}$ = 6.44, p = 0.011; Figure 4.2B) and there was no longer an effect of the interaction with the P0 treatment ($\chi^2_{(1)}$ = 0.34, p = 0.56). Additionally, females housed in groups laid heavier eggs than females housed in pairs ($\chi^2_{(1)}$ = 6.02, p = 0.014; Figure 4.2C) and the F2 offspring of females housed in groups were heavier at hatching than offspring of females housed in pairs ($\chi^2_{(1)}$ = 12.53, p < 0.001, Figure 4.2D). The P0 social environment did not affect growth, mass at day 90, egg mass, or F2 mass at hatching, and did not interact with the effects of the F1 social environment (all $\chi^2_{(1)}$ values < 1.36, all p-values > 0.24, all $\chi^2_{(3)}$ values < 4.51, all p-values > 0.21; Supplementary Data 4.1).

Egg mass was significantly positively correlated with F1 female mass at day 90 ($\chi^2_{(1)}$ = 5.59, p = 0.02; Supplementary Figure 4.1A). When controlling for female mass at day 90, the effect of the female's own social environment on egg mass was no longer significant ($\chi^2_{(1)}$ = 2.45, p = 0.12), suggesting that the effect of the F1 social environment on egg mass was mediated by effects on female body mass. Similarly, F2 mass at hatching was significantly positively correlated with egg mass ($\chi^2_{(1)}$ =135.61, p < 0.001; Supplementary Figure 4.1B), and when controlling for egg mass, the effect of the female's own social environment on F2 mass at hatching was no longer significant ($\chi^2_{(1)}$ = 1.39, p = 0.24). This suggests that the effect of the F1 social environment on F2 mass at hatching was mediated by the effects on egg mass.

4.3.2. Stress protocol and GnRH challenge

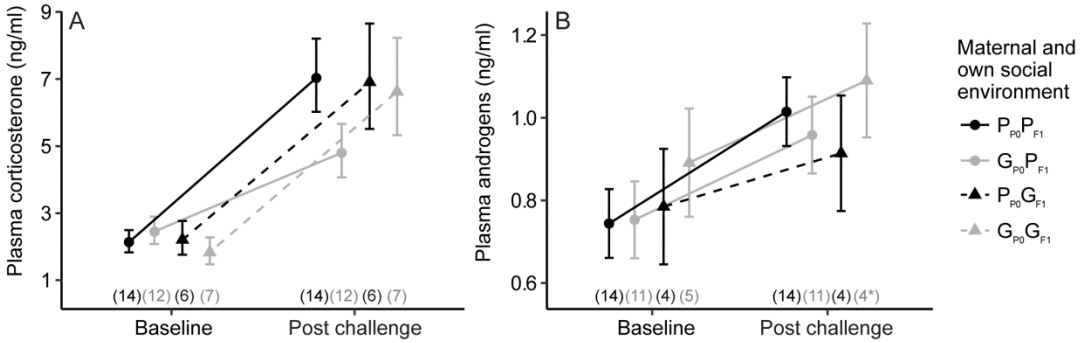

Figure 4.3. A: plasma corticosterone concentrations of F1 females 90-91 days old before and after being restrained for 10 minutes (back-transformed from log10). 10 minutes of restraint significantly increased plasma corticosterone concentrations, but there was no effect of the maternal or own social environment or their interaction on the increase, or on average plasma corticosterone concentrations. B: plasma androgen concentrations of F1 females 96-97 days old before and after an injection with 5 µg GnRH. Androgen concentrations increased significantly in response to the GnRH injection, but there was no effect of the maternal or own social environment or their interaction on the increase, or on average plasma androgen concentrations. Data shown are the estimated means ± 1 SEM. Numbers between brackets indicate sample sizes. *: insufficient plasma for one $G_{P0}G_{F1}$ female in the response sample.

Females responded to the 10 minutes of restraint with a significant increase in plasma corticosterone concentrations ($\chi^2_{(1)}$ = 53.24, p < 0.001; Figure 4.3A), but the corticosterone response did not differ between females from different maternal or own social environments (maternal social environment * sample: $\chi^2_{(1)}$ = 1.69, p = 0.19; own social environment * sample: $\chi^2_{(1)}$ = 1.69, p = 0.19; Figure 4.3A). There was also no effect of the interaction between the maternal and own social environment on the female's stress response (maternal social environment * own social environment * sample: $\chi^2_{(1)}$ = 2.33, p = 0.13; Figure 4.3A). Average plasma corticosterone concentrations were not affected by the female's own social environment, the maternal social environment, or their interaction (all $\chi^2_{(1)}$ values < 0.64, all p values > 0.43 Figure 4.3A; Supplementary Table 5 in Supplementary Data 4.1).

GnRH injections resulted in a significant increase in plasma androgen concentrations ($\chi^2_{(1)}$ = 26.43, p < 0.001; Figure 4.3B), but the androgen response to the GnRH challenge did not differ between females from different maternal or own social environments (maternal social environment * sample: $\chi^2_{(1)}$ = 0.22, p = 0.64; own social environment * sample: $\chi^2_{(1)}$ = 0.96, p = 0.33; Figure 4.3B). The female's androgen response to GnRH was not affected by the interaction between the maternal and own social environment (maternal social environment * own social environment * sample: $\chi^2_{(1)}$ = 0.72, p = 0.40; Figure 4.3B). Average plasma androgen concentrations were not affected by the female's own social environment, the maternal social environment, or their interaction (all $\chi^2_{(1)}$ values < 0.55, all p values > 0.46; Figure 4.3B; Supplementary Table 6 in Supplementary Data 4.1).

4.3.3. Reproduction

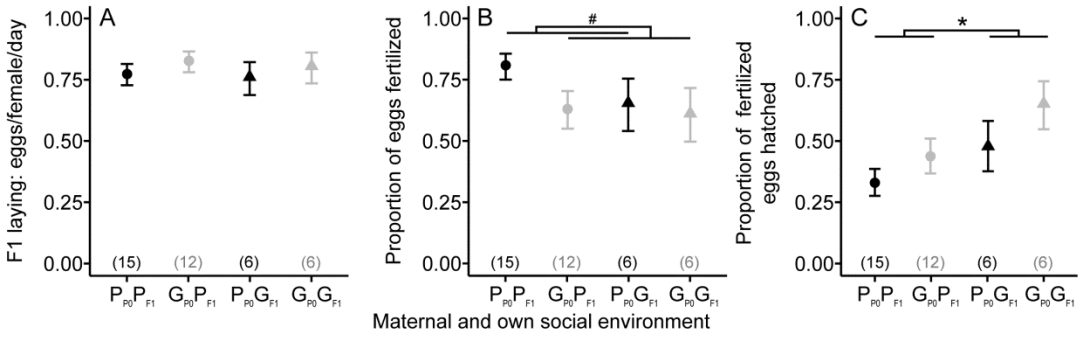

Figure 4.4. A: number of eggs laid per female per day. Egg laying rates were not affected by the maternal or own social environment or their interaction. B: proportion of eggs fertilized. There was a small nonsignificant effect of the maternal social environment, with offspring from pair-housed mothers laying slightly more fertilized eggs than offspring from group-housed mothers. Fertilization success was not affected by the own social environment or the interaction between the maternal and own social environment. C: hatching success of fertilized eggs. Hatching success was higher for females housed in groups compared to females housed in pairs. Hatching success was not affected by the maternal social environment or its interaction with the female's own social environment. Data shown are the estimated means ± 1 SEM (back-transformed from logit). Numbers between brackets indicate the number of F1 females included (for number of eggs, see Table 2).

Table 4.2. Sample sizes for F1 egg laying rates, egg mass, fertilization, hatching success and F2 offspring mass at hatching. a due to an oversight only 77 eggs were weighed. b 21 chicks hatched, but one chick was excluded from the mass measurements because of birth defects.

Maternal and own social environment	F1 females contributing to egg data	Eggs laid	Eggs fertilized	Eggs hatched	F1 females with F2 offspring hatching	F2 offspring
$P_{P0}P_{F1}$	15	93	73	24	13	24
$G_{P0}P_{F1}$	12	79[a]	48	21	8	20[b]
$G_{P0}G_{F1}$	6	38	23	15	5	15
$P_{P0}G_{F1}$	6	36	23	11	4	11

Egg laying rates were not affected by the maternal social environment ($\chi^2_{(1)}$ = 0.89, p = 0.35; Figure 4.4A), the F1 female's own social environment ($\chi^2_{(1)}$ = 0.11, p = 0.75; Figure 4.4A), or the interaction between the maternal and own social environment ($\chi^2_{(1)}$ = 0.01, p = 0.92; Figure 4.4A). Offspring from pair-housed mothers laid slightly more fertilized eggs than offspring from group-housed mothers, but the difference did not reach statistical significance ($\chi^2_{(1)}$ = 2.89, p = 0.09; Figure 4.4B). There was no effect of the F1 female's own social environment ($\chi^2_{(1)}$ = 1.08, p = 0.30; Figure 4.4B) or of the interaction between the maternal and own social environment on fertilization success ($\chi^2_{(1)}$ = 0.77, p = 0.38; Figure 4.4B).

The hatching success of fertilized eggs was higher for females housed in groups compared to females housed in pairs ($\chi^2_{(1)}$ = 4.07, p = 0.04; Figure 4.4C). The maternal social environment and its interaction with the female's own social environment did not affect hatching success of fertilized eggs ($\chi^2_{(1)}$ = 2.63, p = 0.11 and $\chi^2_{(1)}$ = 0.13, p = 0.72, respectively; Figure 4.4C). Overall hatching rates (the proportion of all eggs collected for the F2 generation that hatched, i.e. including non-fertilized eggs) were not affected by the female's own social environment, the maternal social environment, or their interaction (all $\chi^2_{(1)}$ values < 1.88, all p values > 0.17; Supplementary Table 10 in Supplementary Data 4.1).

4.4. Discussion

This study is the first to our knowledge to test for evidence of adaptive maternal effects and the underlying mechanisms in relation to social group size in a match-mismatch experiment across two generations in Japanese quail. Growth of the F1 females was affected mainly by their own social environment, as females housed in groups grew faster and ended up heavier compared to pair-housed females. Notably, however, mass of the F1 females also depended on the interaction between the maternal and own social environment, which was reflected in the positive effect of group housing on female mass only in daughters of P0 pair-housed females. This positive effect on offspring body mass in the mismatched environment, at least for offspring of pair-housed females, contradicts the adaptive hypothesis and suggests a potential silver spoon effect that benefits offspring of pair-housed mothers in the more competitive group environment (Marshall and Uller, 2007; Uller et al., 2013). There was no effect of the P0 social environment on F1 mass before the F1 social treatment started (see also Langen et al., 2018).The fact that an effect of the P0

treatment was only observed in the F1 group environment supports the idea that more competitive or otherwise challenging conditions may be required to detect maternal effects on offspring phenotype (Benowitz-Fredericks et al., 2015; Verboven et al., 2003). On the other hand, the results could be consistent with an adaptive effect if the smaller body mass observed in the matched environments had fitness benefits that were not detected in our experiment (increased growth is not always beneficial; Emmerson, 1997; Metcalfe and Monaghan, 2003; Ringsby et al., 2015; Stamps, 2007). At any rate, our results emphasize the importance of investigating maternal effects under different environmental conditions in the offspring.

The interaction effect of the P0 and the F1 social environment on female mass disappeared by the time eggs for the F2 were collected and, at that point only the positive effect of the current group size on female mass remained. This effect can explain the larger egg size and hatching success and a positive maternal effect on F2 hatchling mass for group-housed females. The positive effects of group-housing on egg mass and offspring mass at hatching can ultimately have important fitness consequences because both are important predictors of offspring growth and survival (Krist, 2011; Williams, 1994). Our results thus strongly suggest that there is additional scope for adaptive maternal effects in relation to group size in Japanese quail and that the observed effects of the social environment on growth have important consequences for egg and offspring quality.

The effects of pair-housing versus group-housing on females and their offspring differed between the P0 and F1 generations. In the P0 generation (Langen et al. 2017), female endocrine physiology was affected but there were no effects on growth, reproduction or F1 offspring mass at hatching. In contrast, the social environment of the F1 females affected growth, reproduction, and F2 offspring mass, but not endocrine physiology. A possible explanation for these differences could be that the sex ratios within pairs and groups differed between the generations. Whereas males were continuously present in the female's social environment in the P0 generation, they were housed separately from the females in the F1 generation, and male-female interaction was only possible during the mating sessions. Pair-housing in the P0 generation likely resulted in more social stimulation by the male, leading to elevated female plasma androgen levels and a trend of higher plasma corticosterone (Langen et al., 2017). This effect by the male might have been diluted in the P0 group environment. In the F1 generation, female exposure to the male was standardized, explaining the absence of a treatment difference in endocrine parameters and a stronger effect of group size on female mass. The contrasting effects of the P0 and the F1 social treatments may not only have been caused by the differences in sex ratio, but also by slight differences in timing between the P0 and F1 generation in the onset of the social treatments (day 29 in the P0 generation vs. day 24 in the F1 generation), the age at which females were first mated, the timing of sampling (for details see Figure 4.1) and the number of females present.

F1 females that were housed in groups grew faster than pair-housed females and were heavier around the time of egg collection. This was unexpected since a negative

correlation between group size or social density and growth has been reported in many animal species, including Japanese quail, likely due to increased competition for resources (Asghar Saki et al., 2012; Keeling et al., 2003; Onbaşılar and Aksoy, 2005). However, increased social stimulation can also lead to increased body mass, as demonstrated in European starlings (*Sturnus vulgaris*; Witter and Goldsmith, 1997), potentially because higher levels of social stimulation can increase food intake rates (Beauchamp, 1998; Hoppitt and Laland, 2008; Tolman, 1964). Increased growth is generally expected to be beneficial under higher social densities because it may increase female competitive abilities (Clutton-Brock and Huchard, 2013; Stockley and Bro-Jørgensen, 2011), and our results indicate that it can lead to increased reproductive investment, in line with previous findings (Christians, 2002; Drent and Daan, 1980; Lim et al., 2014; Ronget et al., 2018; Sockman et al., 2006).

Egg laying rates were not affected by the maternal or the own social environment and fertilization success was not affected by the own social environment, but daughters from pair-housed mothers had a non-significantly higher proportion of fertilized eggs than daughters from group-housed mothers. This effect was small and did not reach statistical significance, but a similar trend to higher fertility of pair-housed mothers was seen in the P0 generation. This suggests a genetic or non-genetic maternal effect on fertility which should be further investigated as it is a core fitness component.

Effects on female mass and reproduction in the F1 generation did not correspond with changes in female endocrine parameters, suggesting that effects of the social environment on female mass and reproduction were not mediated by differences in female plasma androgens and corticosterone in our experiments. Vice versa, in the P0, hormone differences did not lead to reproductive differences. Other studies report non-significant, positive, and negative correlations between circulating androgens or glucocorticoids and measures of reproduction (e.g. egg production: Gerlach and Ketterson, 2013; Veiga and Polo, 2008, hatching success: de Jong et al., 2016; Schmidt et al. 2009, number of fledglings: Burtka et al 2016; O'neal et al. 2008; Ouyang et al., 2011), suggesting that the relationships are non-linear and can change across contexts and over time (Bonier et al., 2009a; Hau and Goymann, 2015; Ouyang et al., 2013, 2011). Moreover, it is important to note that due to the exclusion of some groups as a result of aggression the sample size of group-housed females for the endocrine measurements became rather small at the end of the study when hormone measurements were taken (ranging from four to seven females).

4.5. Conclusions

We have shown that maternal effects of the social environment can depend on the offspring environment, suggestive of either silver spoon or adaptive anticipatory effects in relation to group size. Group housing – most likely the more competitive environment - revealed the effects of the maternal social environment on female body mass, whereas no differences were seen in the presumably less competitive pair environment. The effects of the maternal social environment disappeared over time to be replaced by the effects of the F1 own social environment, which resulted in a maternal effect on the F2 generation that was independent of the P0 social environment. The observed changes in mass in the F1 and

F2 generations are likely to have important consequences for performance and fitness, but their adaptive significance remains unclear. Effects of social group size on female physiology and reproduction differed between the P0 and the F1 generation most likely because the adult sex ratio did not remain constant over the generations. This might have led to differences in social stimulation between pairs and groups of both generations, potentially explaining why the effects of the matched and mismatched social conditions were less clear than expected. Future studies of the adaptive maternal effects of the social environment and the underlying proximate mechanisms should assess the fitness consequences for offspring in more depth. Furthermore, the importance of the type of social stimuli experienced (e.g. group size, adult sex ratio, intrasexual and intersexual interactions) should be investigated in more detail.

Acknowledgements

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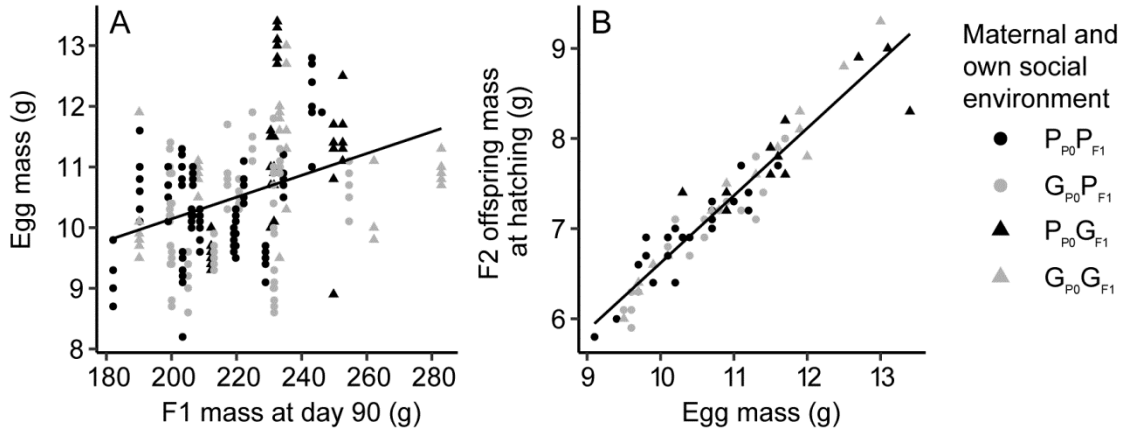
Supplementary material



Supplementary Figure 4.1. A: relationship between F1 female mass around egg collection (day 90) and egg mass. B: relationship between egg mass and mass of the F2 offspring at hatching.

Supplementary Data 4.1. Summaries of all model outputs. To be published with the final article after acceptance. Before publication, data can be requested via E.M.A.Langen@uu.nl

Supplementary Data 4.2. Raw dataset. To be published with the final article after acceptance. Before publication, data can be requested via E.M.A.Langen@uu.nl

Chapter 5

General discussion

5.1. Introduction

An individual's physical appearance, behaviour, and physiology is not only shaped by genes passed on by its parents, but to a large extent also by environmental factors. Environmental influences on phenotypic characteristics of breeding females can even be transferred to the offspring. These non-genetic maternal effects can have important evolutionary consequences, as they affect fitness across generations (Mousseau and Fox, 1998). The social environment, including density, group size and a range of social stimuli, such as agonistic, socio-positive and sexual interactions, constitutes an important environmental factor that can affect breeding females, with consequences for offspring phenotypes, yet the underlying proximate and ultimate mechanisms remain unclear. In addition, whether the social environment may induce anticipatory maternal effects, thus adaptively shape offspring phenotypes with respect to their future environment, lacks scientific evidence (Uller et al., 2013).

This thesis investigated the effects of an important aspect of the social environment – group size, manipulated by housing females in pairs or groups of four – on females and their offspring in a precocial avian species, the Japanese quail (*Coturnix japonica*). Our experiments revealed that the social environment affects female physiology and reproduction, and offspring phenotype (chapters 2 and 4). Furthermore, we show that the maternal and offspring social environments can have interacting effects, which may be an indication for adaptive anticipatory maternal effects (chapter 4). However, the effects of the social treatments on females and their offspring differed between the maternal (chapters 2 and 3) and offspring (chapter 4) generation, and the direction of effects ran counter to our expectations.

Differences in the types of social stimuli used might explain why results vary between studies, including the ones described in this thesis. Furthermore, the timing of measurements and manipulations of the social environment and phenotypic aspects, both in mothers and their offspring is likely to affect the outcome of studies investigating maternal effects of the social environment. This chapter will expand upon these ideas and provide an integrative discussion of the main findings of this thesis.

5.2. Effects of the social environment on female physiology and reproduction

In the parental generation (P0, chapter 2), we expected group housing to lead to higher plasma androgen and corticosterone levels, based on previous studies on the effects of increasing social densities and social challenges (Cain and Ketterson, 2012; Guibert et al., 2010; Ketterson et al., 2005; Langmore et al., 2002; Mazuc et al., 2003; Nephew and Romero, 2003; Rutkowska et al., 2011; Smith et al., 2005). However, while the social environment indeed affected P0 female endocrine physiology, the direction of the effects ran counter to our expectation as in our study, pair-housed females had elevated plasma

androgen (testosterone and 5-α-dihydrotestosterone) and tended to have higher plasma corticosterone concentrations during reproduction compared to group-housed females.

We hypothesized that the lower male to female ratio in groups compared to pairs could explain the effect on female plasma hormone levels (chapter 2). Pair-housed females were likely exposed to increased stimulation by the male, which might have caused the higher levels of circulating androgens and corticosteroids compared to group-housed females. Several studies suggest that intersexual interactions can have profound effects on female endocrine physiology, causing increases in circulating androgens or glucocorticoids (e.g. Correa et al., 2011; Marshall et al., 2005; Rutkowska et al., 2011). Male Japanese quail are known for a high drive to copulate, at least in captivity, and forced copulation has been suggested as a source of stress for the females (Adkins-Regan, 2015, 1995; Galef Jr., 2008; Rutkowska et al., 2011), which may explain increases of female plasma corticosterone concentrations in response to copulation (Correa et al., 2011). In addition, increased levels of circulating androgens in response to copulation have been reported, which also strongly depends on male body condition and the intensity of male copulatory behaviour (the number of times a male mounts the female; Correa et al., 2011). Though we were not able to disentangle the effects of group size and male-female interactions, our hypothesis that the amount of stimulation by the male caused the reported differences in female endocrine physiology was further supported by the finding that more bald females (baldness is caused by repeated copulation with the male: Kovach, 1974; Mills et al., 1997; chapter 2), had higher baseline plasma androgen and corticosterone levels. Additional support comes from the result that the social treatment of the adult F1 females, where pairs and groups consisted of only females, did not induce differences in female androgen and corticosterone levels (chapter 4). Instead, the differences in the number of conspecific females in a cage affected female growth and reproductive investment. Females from both F1 social environments received similar amounts of stimulation from the males during the mating sessions, and by mating females from the four P0-F1 treatment combinations with the same male, potential differential effects of individual males on females and offspring investment (e.g. Alonso-Alvarez et al., 2012; Correa et al., 2011; Cunningham and Russell, 2000; Petrie and Williams, 1993; Uller et al., 2005) were largely controlled for.

Despite the elevated hormone levels in P0 pair-housed females, the endocrine response to standardized challenges (restraint stress protocol and GnRH challenge) did not differ between pair-housed and group-housed females (chapter 2), suggesting that the sensitivity of the hypothalamic-pituitary-adrenal (HPA) and the hypothalamic-pituitary-gonadal (HPG) axis were not affected. Also in the F1 generation, we found no indication for an effect of the social environment on the responsiveness of the HPA and HPG axis (chapter 4). Thus, while previous studies have shown that social stimuli may affect the stress response (HPA; DeVries et al., 2003; Scheiber et al., 2009) and thereby reproduction (HPG), our results do not confirm these findings. However, both the HPA and HPG axis are complex endocrinological systems, and changes could occur on several levels, which were not investigated in our studies, including regulation of brain receptors (Canoine et al., 2007;

Harris et al., 2013), carrier proteins (Deviche et al., 2001; Malisch and Breuner, 2010; Schoech et al., 2013), clearance rate/recovery to baseline (Breuner et al., 1999; Ericsson et al., 2014; Zimmer et al., 2013), and behaviour (Careau et al., 2014).

In the P0 generation, female endocrine physiology was affected by the social environment, with no significant effects on reproduction (chapter 2), whereas the social environment of the F1 females affected reproductive investment, but not endocrine physiology (chapter 4; see Table 5.1 for an overview of the differences in effects). Thus, although steroids are important regulators of reproduction, effects of the social environment on female reproduction were not likely to be caused by differences in female plasma androgens and corticosterone in our experiments. However, as described in chapter 1, the effects of increasing hormone concentrations may be highly time- and context-dependent and non-linear (Bonier et al., 2009; Hau and Goymann, 2015; Ouyang et al., 2013, 2011). Negative relationships between circulating androgen and corticosterone concentrations and reproduction might only show at the more extreme ends of the physiological range (either very low or very high levels may compromise reproduction) and intermediate changes in circulating hormone concentrations in the P0 females might not have been sufficient to affect reproduction. Nevertheless, since many more physiological parameters that we did not measure could be of importance, we cannot exclude that the changes in plasma hormones may have, in the long term, affected lifetime reproductive success.

Table 5.1. Overview of effects of the female's own social environment in the P0 (chapter 2) and F1 (chapter 4) generation. Arrows indicate the direction of effects (arrows in between brackets indicate a nonsignificant trend; p = 0.05-0.1)

		Generation	
Measure		**P0**	**F1**
Growth		Pair = Group	Pair < Group
Egg production (eggs/female/day)		Pair (>) Group	Pair = Group
Fertilization success		Pair (>) Group	Pair (>) Group
% all eggs hatched		=	=
% fertilized eggs hatched		na	Pair < Group
Egg mass		=	Pair < Group
Hatchling mass		=	Pair < Group
Plasma corticosterone concentrations			
	Baseline	Pair (>) Group	Pair = Group
	Post-challenge	Pair (>) Group	Pair = Group
Plasma androgen concentrations			
	Baseline	Pair > Group	Pair = Group
	Post-challenge	Pair > Group	Pair = Group

While the social environment of the adult F1 females did not affect their circulating androgen and corticosterone levels, there were significant effects on their growth and reproductive investment (chapter 4). Despite ad libitum feeding in both social

environments, group-housed F1 females grew faster than pair-housed females, were heavier around the time of egg laying, produced heavier eggs with a higher hatching success, and had heavier F2 offspring hatching. At first view, the fact that group-housing caused increased growth might be counter-intuitive, as in many animal species, including Japanese quail, a negative correlation between group size or social density and growth has been reported, likely due to increased competition for resources (Asghar Saki et al., 2012; Bonenfant et al., 2009; Keeling et al., 2003; Onbaşılar and Aksoy, 2005). However, it may be that the relationship between group size and growth is non-linear, being positive for smaller group sizes, while turning negative when group size becomes too large. Our results are in line with findings in European starlings (*Sturnus vulgaris*), where increased social stimulation can also led to increased body mass (Witter and Goldsmith, 1997). In F1 groups, growth might have been increased due to social facilitation of feeding behaviour (Beauchamp, 1998; Hoppitt and Laland, 2008; Tolman, 1964) or changes in activity patterns (Leone and Estevez, 2008), affecting the female's metabolism.

Ultimately, increased growth can be beneficial under higher social densities as it could increase the competitive abilities of an individual (Clutton-Brock and Huchard, 2013; Stockley and Bro-Jørgensen, 2011). Furthermore, increased growth could have important reproductive benefits as heavier females may be able to invest more in reproduction (Christians, 2002; Drent and Daan, 1980; Lim et al., 2014; Ronget et al., 2018; Sockman et al., 2006). Indeed, the fact that group-housed females were not only heavier, but also produced heavier eggs with a higher hatching success and had heavier F2 offspring hatching indicates that the effects of the F1 social environment on growth can have consequences for offspring investment.

The morphological effects on the F1 females were transmitted to their offspring, with effects lasting at least until the hatchling stage. The next sections will focus on some of the potential pathways through which prenatal maternal effects are mediated.

5.3. Effects of the social environment on egg composition and offspring phenotype

A main pathway of maternal effects is the transmission of resources to the developing offspring. The amount and quality of resources provided by the mother depends to large extent on her physiology (e.g. circulating hormone levels) and morphology (e.g. body mass), factors that can be affected by variation in the social environment, as we have also shown in chapters 2 and 4. During breeding, changes in the maternal phenotype in response to social stimuli are therefore likely to result in maternal effects on egg composition, with consequences for offspring phenotype.

5.3.1 Egg mass

In both the P0 and F1 generation, we investigated the effects of the social environment on egg mass, being an indicator of maternal nutrient provisioning, and an important predictor

of offspring size and/or survival (Bernardo, 1996; Krist, 2011; Williams, 1994). In the P0 generation, eggs from pair-housed versus group-housed females did not differ in mass, potentially explained by the fact that the social environment did not affect female mass in the P0 generation (chapter 2). Reproductive investment is often positively correlated with female mass or size (Christians, 2002; Drent and Daan, 1980; Lim et al., 2014; Ronget et al., 2018; Sockman et al., 2006; Verboven et al., 2003), as was also shown in the F1 generation, where the increased growth and body mass of group-housed females corresponded with heavier egg mass and subsequently F2 offspring mass at hatching (chapter 4). Since egg mass and hatch mass are often a positive predictor of subsequent offspring growth and survival, the effects reported in the F1 generation could have important fitness consequences (Bernardo, 1996; Krist, 2011; Williams, 1994). Follow-up studies on the F2 generation should investigate whether the higher hatchling mass of F2 offspring from F1 females housed in groups indeed results in increased growth and survival, and whether this positively affects their future reproductive output (Langen et al., in prep).

5.3.2 Yolk testosterone

Next to egg mass, another important mediator of maternal effects is prenatal exposure to maternally derived hormones (Groothuis et al., 2005). Against our predictions and despite differences in plasma steroid levels, we did not find effects of pair-housing versus group-housing on yolk testosterone levels in the P0 generation. In a range of avian species, the amount of social stimulation (e.g. breeding density, social instability or female-female competition) has been positively correlated with yolk androgen concentrations and yolk testosterone in particular (Bentz et al., 2018, 2016, 2013; Eising et al., 2008; Guibert et al., 2010; Hargitai et al., 2009; Mazuc et al., 2003; Pilz and Smith, 2004; Reed and Vleck, 2001; Schwabl, 1997; Whittingham and Schwabl, 2002). However, contrasting results have also been reported, finding no or negative correlations between social competition and yolk testosterone levels, indicating that interspecific variation is high (Bentz et al., 2016). Furthermore, differences between studies in the type of social stimuli used could again explain why results differ. For example, while social instability has been shown to increase yolk testosterone deposition in Japanese quail (Guibert et al., 2010), our results indicate that differences in social group size have no effects (chapter 2).

Moreover, we cannot exclude effects of the social environment on other egg components, which likely did occur as the P0 social environment affected F1 female growth at later ages (after day 24; chapter 4). While this thesis focuses predominantly on yolk testosterone as an endocrine mediator of maternal effects, yolk contains many more hormones, including androstenedione, dihydrotestosterone, progesterone, estradiol and corticosterone (Almasi et al., 2012; Rettenbacher et al., 2009; Schwabl, 1993; von Engelhardt and Groothuis, 2011). In addition to steroids, also thyroid hormones (Ruuskanen et al., 2016; Ruuskanen and Hsu, 2018), antioxidants (Blount et al., 2000; Surai et al., 2001, 1998; Surai and Speake, 1998) or immune substances (Buxton, 1952; Hasselquist and Nilsson, 2009; Kowalczyk et al., 1985) are deposited in the yolk of the developing egg. Not only are these different egg traits likely to affect offspring development in different ways,

their effects may also interact (Giraudeau et al., 2017). In order to gain better understanding of what mediates maternal effects, the relative importance of factors such as egg size, yolk hormones, immune substances or antioxidants for offspring development should be further investigated.

Several studies have suggested that yolk and maternal plasma androgen concentrations are independently regulated (reviewed in Groothuis and Schwabl, 2008), and that yolk testosterone predominantly originates from the follicular walls surrounding developing oocytes (Hackl et al., 2003; Okuliarová et al., 2010). Androgen production in the cells of the follicular walls can be stimulated by GnRH (via luteinizing hormone – LH – and follicle stimulating hormone - FSH). The magnitude of the androgen response to GnRH has been suggested as a better predictor of yolk testosterone deposition than baseline plasma concentrations (Jawor et al., 2007; Müller et al., 2011; Peluc et al., 2012). In the P0 generation, however, the androgen response to GnRH did not predict yolk testosterone levels, neither did baseline plasma androgen levels (chapter 2). Our results thus support the independent regulation hypothesis, though the timing of yolk testosterone measurements and the GnRH challenge could explain differences between our study and others. Studies that found a correlation between GnRH responsiveness and yolk testosterone, measured yolk testosterone in subsequently laid eggs, whereas we challenged our P0 females with GnRH after the eggs for yolk hormone measurement were collected. Furthermore, the female's GnRH response was smaller than previously reported for Japanese quail, in response to a similar dosage of GnRH (chicken GnRH-I; Peluc et al., 2012). A low response to GnRH may not be reflected in yolk testosterone levels, as opposed to a high GnRH response. Finally, a recent study by (Okuliarova et al., 2018) suggested that the peak in LH before ovulation is related to yolk testosterone deposition. Here, females that were selected for high yolk testosterone concentrations showed a higher LH peak than females selected for low yolk testosterone concentrations, but the selection lines did not differ in their plasma testosterone levels or their LH or androgen response to GnRH. While this again suggests that yolk testosterone deposition is reflected in the activity of the HPG axis, it also suggests that differences in HPG axis activity are not necessarily accompanied by an increased LH or androgen responsiveness to GnRH.

5.3.3 Offspring phenotype

We have shown that the effects of the social environment on female mass in the F1 resulted in maternal effects on egg mass and F2 offspring mass at hatching. This indicates that social influences on female body mass can be transmitted to the offspring, egg mass being an important mediator of this effect.

The social environment of the P0 females did not induce maternal effects on F1 juvenile growth, survival and plasma concentrations of androgens and corticosterone (chapter 3). This is perhaps not surprising given that the P0 social environment did also not affect yolk testosterone, yolk mass and egg mass, all important mediators of effects on offspring growth and survival (Matson et al., 2016; Müller et al., 2007; Navara et al., 2005,

2006; Okuliarova et al., 2011; Possenti et al., 2016; Schwabl, 1996b; Sockman and Schwabl, 2000) or physiology (Daisley et al., 2005; Müller et al., 2007; Pfannkuche et al., 2011). Along these lines, we also did not find any significant effects of the P0 social environment on F1 juvenile tonic immobility (TI) or emergence behaviour (Langen et al., in prep; see Box 1), which have previously been show to correspond with changes in yolk testosterone levels (Daisley et al., 2005; Guibert et al., 2010; Okuliarová et al., 2007). Our results suggest again that differences in the type of social stimuli can lead to different effects on egg composition and offspring phenotype, as the effects of maternal social group size in our experiments differed from the effects of maternal social instability described in an earlier study on Japanese quail (Guibert et al., 2010). Here, offspring from mothers in an unstable social environment were slower to emerge than offspring from mothers in a stable social environment.

While we did not detect maternal effects on F1 juvenile phenotypes, effects of the social environments emerged after adult F1 females entered their own social treatment (chapter 4). Adult body mass of the F1 females was affected by the interaction between the maternal and own social environment: group-housed daughters of pair-housed mothers were heavier females with a different P0-F1 treatment combination. The maternal effects on F1 body mass only emerged when offspring were exposed to their adult social environments, and only in F1 groups. Since the F1 juveniles were also kept in groups, one could wonder why the effects of maternal pair housing on F1 mass did not emerge sooner. Likely, juvenile groups comprise very different social stimuli than adult groups, as social behaviour develops and changes during maturation (François et al., 1998). Furthermore, effects on growth and body mass might only emerge at certain ages (Monaghan, 2008) due to age-specific differences in genes regulating these traits (Johnsson et al., 2018).

5.3.4 Offspring sex allocation

Apart from shaping offspring phenotype, changes in maternal hormones may even lead to skews in offspring sex ratio - the proportion of sons and daughters (Alonso-Alvarez, 2006; James, 2008; Krackow, 1995; Navara, 2013a; Pike and Petrie, 2003). However, we found no indication that the effects of the social environment on female physiology in the parental generation resulted in changes in offspring sex ratio at any stage (embryonic, at hatching and at 23 days of age, chapter 3). Previous studies in avian species have suggested a general pattern that increased maternal androgen levels lead to male-biased offspring sex ratios (Goerlich-Jansson et al., 2013; Goerlich et al., 2009; Pike and Petrie, 2005; Rutkowska and Cichoń, 2006; Veiga et al., 2004) while elevated maternal corticosterone levels lead to female-biased offspring sex ratios (Bonier et al., 2007; Goerlich-Jansson et al., 2013; Love et al., 2005; Pike and Petrie, 2006, 2005). In Japanese quail, increased maternal corticosterone has been linked to a female-biased offspring sex ratio (Pike and Petrie, 2006), while for maternal androgens, various directions of effects have been reported, as they have been linked to both a female-biased (Correa et al., 2011) as well as an unbiased offspring sex ratio (Pike and Petrie, 2006). The variability of outcomes demonstrate that we still lack

knowledge on the mechanisms underlying sex ratio adjustment, warranting further research.

As described in chapter 3, differences between studies that might explain contrasting results include the timing of measurements and manipulation of both endocrine and social parameters. Influences of the maternal environment and physiology on developing offspring may only occur during critical windows (Okuliarova et al., 2018), which has already been suggested for sex ratio adjustment, where maternal steroids may influence the segregation of the sex chromosomes only during the first meiotic division (Correa et al., 2005; Goerlich-Jansson et al., 2013; Navara, 2013a, 2013b; Pinson et al., 2011; Rutkowska and Badyaev, 2008). The lack of significant patterns in our study may be explained by not measuring maternal hormones during the time window during which genetic sex determination takes place (meiosis I). Furthermore, other steroids than maternal androgens and glucocorticoids may be involved in biasing offspring sex ratio (e.g. progesterone (Correa et al., 2005).

5.4. Timing of maternal effects

As mentioned in section 5.3, there are many different pathways through which prenatal maternal effects can be established and different factors can have interacting effects. Next to investigating what mediates maternal effects by studying the relative importance of factors such as egg size, yolk hormones, immune substances or antioxidants for offspring development, it is important to investigate at which times maternal effects are likely to manifest. Maternal influences on the egg or offspring phenotype might only take place during sensitive windows. In section 5.3, as well as in chapter 3, we have already discussed that effects of maternal hormones on offspring sex ratio might only take place during the first meiotic division. Another example of a sensitive window for maternal effects is the phase of rapid yolk deposition, a period during which maternal effects on yolk composition are likely the strongest (Groothuis et al., 2005; Groothuis and von Engelhardt, 2005). Measuring or manipulating the maternal environment or physiology during these sensitive windows can be crucial for finding an effect. Not only when, but also how long and how often measurements and manipulations take place are important factors to consider. In some cases, investigating maternal effects on eggs or offspring phenotype might require integrated measure of maternal hormones over a longer time period (for example, during the phase of rapid yolk deposition which spans across multiple days). Also in the offspring, the timing of physiological measures can have a strong impact on results. Circulating hormone levels change during the maturation of the endocrine axis and variation can be very high at certain ages, which complicates finding any effect of maternal influences (Hazard et al., 2005; Ottinger et al., 2001).

As discussed in chapter 4, we cannot rule out the possibility that differences between the P0 and F1 generation in the effects of pair-housing versus group-housing were caused by slight differences in the onset of the social treatments (at the age of 29 days in the P0 generation, versus 24 days in the F1 generation), the age at which adult females first encountered a male (at 29 days of age, before reaching sexual maturity in the P0

generation, or at 55 days of age in the F1 generation), or the timing of sampling (see chapter 4, Figure 4.1 for details). The influence of social stimuli on an individual's phenotypic development likely vary across life stages, and, like maternal influences might depend on sensitive windows (e.g. early life: Naguib et al., 2011, or adolescence: Bölting and von Engelhardt, 2017; Ruploh, 2014).

5.5. The adaptive significance of maternal effects of the social environment

In chapter 4, adult F1 females were housed under social conditions that either matched or mismatched their maternal social conditions with respect to group size (pairs of two females and groups of four females). The maternal social environment affected F1 female mass in a context-dependent way, indicating a match/mismatch effect: offspring from pair-housed mothers were heavier when they were housed in groups themselves, compared to pair-offspring housed in pairs. Together with other studies reporting context-dependent maternal effects (e.g. Benowitz-Fredericks et al., 2015; Giordano et al., 2014; LaMontagne and McCauley, 2001; Plaistow and Benton, 2009; Plaistow et al., 2006), our results emphasise once more the importance of investigating maternal effects under different environmental conditions in the offspring.

The result that 'mismatched' group-housed daughters from pair-housed mothers were heaviest on average contradicted our initial expectations. Offspring housed under social conditions that were matched to their maternal social conditions (with respect to group size) were expected to perform better than offspring housed under mismatching social conditions, and we therefore predicted increased growth and fecundity. As discussed in section 5.2, variation in the adult sex ratios between pairs and groups of the P0 and F1 generation likely affected the amount and type of social stimulation that individual females received in groups or pairs. This could have affected the degree to which the offspring environments could match the maternal social environments. Whereas in the P0 generation, the pair environment might have resulted in more stimulation (by the male), in the F1 it is likely the group environment where social stimulation was increased (by the higher number of females compared to pairs). Pair-housed mothers, experiencing high levels of sexual stimulation by the male, might have prepared their female offspring for a similar environment by increasing female offspring body mass (in Japanese quail, larger females have been suggested to have more control over intersexual interactions; Correa et al., 2011). However, since in the F1 generation, the more stimulating environment was likely the group environment, daughters from pair-housed mothers might have benefited more from the maternal effects in the group environment compared to the pair environment, revealing the effects of maternal pair-housing only in groups. This idea that maternal effects on offspring phenotype may only be revealed under more competitive or otherwise challenging conditions, has also been suggested in previous studies (Benowitz-Fredericks et al., 2015; Verboven et al., 2003). For example, in common murres (*Uria aalge*), prenatal exposure to glucocorticoids positively correlated with juvenile circulating

glucocorticoid levels, but only after imposing the challenge of food restriction (Benowitz-Fredericks et al., 2015). In great tits, maternal food restriction reduced offspring body mass and tarsus length, but only under food restricted conditions, and under non-restricted conditions, maternal food restriction tended to increase offspring size (Giordano et al., 2014). To test the hypothesis that the effects of P0 pair-housing were revealed in F1 groups due to increased competition, future studies could benefit from more detailed observations of social relationships within pairs or groups, which could provide important information regarding the amount and type of social stimulation.

Notably, increased growth may also have detrimental effects on for example telomere length (Ringsby et al., 2015) and may negatively affect survival (Metcalfe and Monaghan, 2003; Stamps, 2007). Thus, even though increased growth led to an increase in reproductive investment and heavier F2 offspring (chapter 4), there may be negative effects on the female's lifetime reproductive success, which was not measured in our studies.

The adaptive benefits of the context-dependent effects of maternal pair-housing remain unclear, especially because the effects of the maternal social environment appeared to be overridden by the effects of the F1 own social environment. Only the effects of the own social environment on mass were detectable at day 90, and only the own social environment affected egg mass, hatching success and F2 offspring mass at hatching. These effects of the F1 social environment on F2 mass at hatching could be adaptive, since heavier offspring from group-housed F1 females are likely better prepared for a more competitive environment (Clutton-Brock and Huchard, 2013; Stockley and Bro-Jørgensen, 2011) and might have increased survival and growth (Krist, 2011; Williams, 1994). These assumptions remain to be investigated by studying the development, growth, survival and reproductive output of the F2 offspring (Langen et al., in prep).

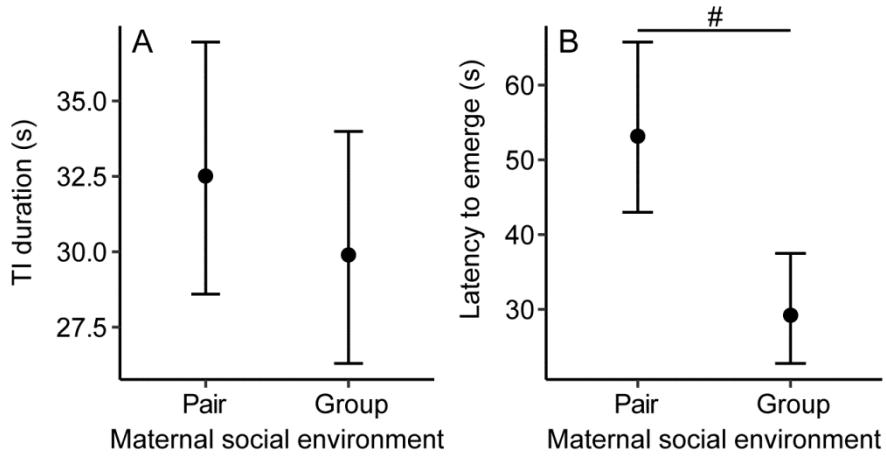
5.6. Concluding remarks and future perspectives

The results from this thesis show that variation in group size can affect female endocrine physiology, growth and reproductive investment. Furthermore, our results show that there can be interacting effects of the maternal and offspring social environment. However, the effects of the social environment on females and their offspring differs strongly between studies, including the studies described in this thesis. Different types of social stimuli likely differ in their effects on female physiology, reproduction, and offspring phenotype. To gain a better understanding of the underlying mechanisms and the function of maternal effects of the social environment, it is therefore important to establish which social stimuli are most important for which effects, and how they interact with each other. The studies described in this thesis point towards a number of factors that should be further investigated, in particular the effects of different adult sex ratios on females and their offspring. Furthermore, more detailed observations of social relationships within pairs or groups could provide important information regarding the amount and type of social stimulation and their effects.

We cannot be certain what caused the context-dependent effects of maternal pair-housing on the F1 females, and the effects eventually appeared to be overridden by the effects of the F1 own social environment. It therefore remains to be determined whether the observed maternal effects represent adaptations. The social environment in the F1 generation affected F2 offspring's mass at hatching, which could be adaptive, but this requires further investigation. Follow-up experiments should test the performance (e.g. growth, survival and reproductive success) of the F2 offspring.

Finally, to understand the mechanisms of maternal effects it is important to gain better understanding of how they are mediated and at which time they manifest. Taking detailed and integrative measures of maternal physiology over time, and correlating them with measures of egg composition or offspring phenotype could provide valuable information regarding the effects of maternal environmental factors and the sensitive time windows during which they can occur. This includes studying how resources accumulate in the yolk, and how environmental factors can influence these processes. In addition, focusing on the effects of a range of egg qualities such as size, various yolk hormones (e.g. progesterone, androstenedione and estradiol; Langen et al. in prep), immune substances or antioxidants simultaneously could give some insight into their relative importance for offspring phenotype.

Box 1. Effects of the maternal social environment on F1 behaviour.



F1 offspring from pair-housed and group-housed mothers were tested for their tonic immobility (TI) response at the age of 16-17 days and their latency to emerge from a box into a novel environment at the age of 22 days. The TI response is thought to be an adaptive anti-predator response, and is used as a measure of fearfulness, with more fearful animals staying in a state of TI for a longer time than less fearful animals (Mills and Faure, 1991; Thompson et al., 1981). The latency to emerge into an open arena is a measure of fearfulness or exploratiVeness. More fearful birds will take longer to emerge, whereas more explorative chicks will have shorter latencies to emerge (Archer, 1973; Jones et al., 1982).

For both tests, one chick at a time was removed from its home cage and transported to the experimental room in a cardboard box. Chicks from the same cage were never tested directly after each other.

TI was induced by placing birds on their back in a u-shaped cradle, and restraining them by placing a hand over the chicks' sternum for 10 seconds. We noted the number of induction attempts needed to achieve TI, and subsequently measured the duration of TI for a maximum of 10 minutes. Chicks that did not get induced within 3 TI induction attempts were considered not inducible and got a score of 0 seconds. Chicks that did not come out of TI within 10 minutes got a maximum score of 600 seconds.

For the emergence test, birds were placed in a closed box (13 × 13 × 15 cm) and left to acclimatize for 30 seconds. After acclimatization, a door at the front of the box was opened, facing an open arena (85 x 80 cm; floor covered with waved cardboard). We recorded the latency to leave the box for a maximum of 10 minutes. Chicks that did not leave the box within 10 minutes got a maximum score of 600 seconds.

The maternal social environment did not affect offspring TI duration (LMM with a fixed effect of the maternal social environment and random effects of maternal cage and mother within maternal cage; $\chi^2_{(1)} = 0.21$, $p = 0.65$; figure A). There was a nonsignificant trend for offspring emergence latency to be affected by the maternal social environment, with offspring form pair-housed mothers taking longer to emerge (LMM with a fixed effect of the maternal social environment and random effects of maternal cage and mother within maternal cage; $\chi^2_{(1)} = 3.01$, $p = 0.08$; figure B). Data shown are the estimated means ± 1 SEM (back-transformed from natural log).

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Addendum

Declaration of originality/Selbstständigkeitserklärung

I hereby confirm that I am aware of the doctoral degree regulations of the University. By presenting this thesis in fulfilment of the requirements for a doctoral degree (Dr. rer. nat.) at Bielefeld University, I declare that this work is original and has not been submitted for a higher degree to any other university or institution. I confirm that I have written this thesis by myself and that I have referenced all support and sources used.

Esther Michelle Alicia Langen
May 2018

Hiermit versichere ich, dass mir die geltende Promotionsordnung der Universität Bielefeld bekannt ist. Dazu versichere ich dass die vorliegende Arbeit zur Erlangung des Doktortitels (Dr. rer. nat.) der Universität Bielefeld ein Originalwerk ist und nicht an einer anderen Universität oder einem anderen Institut zur Erlangung eines höheren Abschlusses eingereicht wurde. Ich versichere, dass ich die vorliegende Dissertation selbstständig verfasst habe, und dass ich alle benutzten Hilfsmittel und Quellen kenntlich gemacht habe.

Esther Michelle Alicia Langen
Mai 2018

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