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Don't underestimate father

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Chapter 6

Synthesis (General Discussion)

Asmoro Lelono

Introduction

Reproductive strategies involve decision making about when and with whom to mate, how much offspring to produce, and how much resources to allocate to offspring in order to maximize current and future reproductive success. When females of similar reproductive potential end up with mates of different quality, a dichotomy in choice emerges for females: Either invest more in current offspring when mated with high-quality males, because the offspring will be of high-quality too (differential allocation (DA) hypothesis, Burley, 1986; Sheldon, 2000), or invest more in current offspring when mated with low-quality males to compensate the offspring (the compensation hypothesis, Gowaty, 2008; Gowaty et al., 2007). Both decisions trade-off current reproduction against future reproduction. The strategy chosen may depend on several aspects, among which the species-specific division of labour between the sexes in raising offspring (Horvathova et al., 2012). Currently, these two hypotheses are now mostly referred to as positive (extra investment) and negative DA (compensation), where positive DA seems to be most common in birds (Haaland et al., 2017; Harris and Uller, 2009; Kindsvater and Alonzo, 2014).

The context-dependent maternal investment, affecting offspring traits, is a maternal effect. Maternal effects are now being recognized as being much more important than previously thought and have received, and still receive, a lot of attention since the stimulating book by (Mousseau and Fox 1999). Egg-laying species such as birds are frequently used for studying maternal effects as a maternal investment in eggs can be quantified relatively easily while bird species show a lot of variation in modes of parental care. Most of the focus has been on the females' side of decision making because generally, they invest more than males in producing offspring: from producing a clutch, incubation, to rearing chicks (Horvathova et al., 2012; Reed and Clark, 2011). In species with female-only care, although they do not directly contribute to paternal care, males can still influence offspring quality other than by their genes (Moller, 2000; Wesolowski, 1994). For example, by increasing their secondary sexual characters, males might enhance their attractiveness for the other sex that in turn may affect maternal investment such as producing larger eggs and allocating more yolk T (e.g. in peahens (*Pavo cristatus*) (Loyau et al., 2007), accumulating higher yolk T in grey partridge (*Perdix perdix*) (Garcia-Fernandez et al., 2010), laying earlier and producing significantly more eggs in the red-legged partridge (*Alectoris rufa*) (Alonso-Alvarez et al., 2012).

This thesis deals with some somewhat neglected aspects of parental effects. I studied if and also how male quality affects maternal reproductive decisions, focussing on endocrine parameters as an underlying pathway, I analysed not only the effect of male visual traits but also on the cryptic effect of ejaculate composition, sex-specific effects on the offspring, and potential costs of maternal egg investment by depositing egg hormones. My study species was the red junglefowl, the wild ancestor of all domesticated chicken strains.

In red junglefowl, the comb size is a males secondary sexual character that reflects quality (Parker, 2003; Parker and Garant, 2004; Parker and Ligon, 2007;). Comb size is dependent on T which can be immunosuppressive (Folstad and Karter, 1992; Zuk, 1995) so in a competitive situation, only males of good-quality can afford to increase their comb size. Expressions of secondary sexual traits in red junglefowl such as body mass, tarsus length, and comb size can be heritable (Parker, 2003; Parker and Garant, 2005; Parker and Ligon, 2007), reflecting genetic variance relevant to fitness, thus favouring females who choose mates on the basis of such traits (Rowe and Houle, 1996). Female red junglefowl are indeed showing a preference for conspecific males with relatively large-comb (Parker and Ligon, 2003).

In order to investigate the relationship between male attractiveness and female reproductive decision making, I used, as a first step, comb size as a signal of quality to study whether females differentiate their reproductive investment. Firstly, I paired up hens with a large or small-comb rooster and allowed them to produce a clutch and raise their offspring. I subsequently paired them up again but now with a male of opposite comb size (chapter 2) and again allowed them to raise a brood. I predicted that females would produce heavier eggs, bigger clutches, and heavier chicks when mated with large-comb males. In fact, I found that hens paired with large-comb males initiated egg laying sooner, but did not lay heavier eggs or produced larger clutches. These first findings fit the positive DA hypothesis because breeding earlier in the season generally yields better results and a higher chance to produce another brood in a single breeding season. I also found that hens mated with large-comb males produced heavier daughters, but lighter sons at five weeks of age) compared to offspring from small-comb males.

The large and bright red coloured comb is a strong visual signal for females on which they base reproductive decisions on (Zuk 1990). Parker (2003) suggested that there may be another pathway by which males may influence reproductive decisions of their mate. He suggested that male quality may be reflected in the composition

(chemical compounds) of his ejaculate. Although Parker (2003) did not explore this any further, it is now known that the ejaculate contains many substances with different functions such as nutrients, minerals, antibodies, and hormones. There are several steroid hormones present in the avian ejaculate such as progesterone, dihydrotestosterone and T (Anderson and Navara, 2011). These hormones are of special interest in this respect since they have a clear signal function, which suggests that they convey 'extra' information from males to females and are a potential way to influence female reproductive decision making. I, therefore firstly studied whether large-comb males had a different ejaculate hormone composition than small-comb males. My focus was on androgens and then specifically T, since this hormone regulates male comb characteristics, male social status, is involved in sperm motility, and moreover females allocate substantial amounts of this hormone to their eggs. I set up several dyadic agonistic encounters between large-comb and small-comb males and observed whether the large-comb males became dominant over the small ones, which was always the case. I then took a blood and ejaculate sample and determined T in both. As expected, the large-comb dominant males had higher plasma T after the encounter, but surprisingly had lower ejaculate T compared to the subordinate small-comb males.

Based on these differences, I then artificially inseminated females with ejaculates containing different T concentrations and measured their reproductive output. In order to achieve this, I collected and pooled ejaculates from several large-comb dominant males. I enriched half of this pool with T (suspended in sesame oil) to the level I previously found in the subordinate males, the other half served as control (I added the T vehicle only). Females were inseminated with either control ejaculates or enriched ejaculates and left to produce two clutches in a cross-over design. I hypothesized that females would invest more, e.g. lay heavier eggs, when inseminated with the ejaculate of dominant males (with a low ejaculate T concentration) and less (lighter eggs) when inseminated with ejaculates mimicking ejaculates from subordinate males (higher ejaculate T concentration). Indeed I found that females inseminated with T enriched ejaculates produced larger eggs compared to when they received control ejaculates.

Furthermore, offspring growth was affected in a sex-specific way. Sons produced from T enriched ejaculates (mimicking small-comb subordinate males) grew slower than sons from control ejaculates, whereas the opposite was true for daughters. This result is in line with the results of offspring growth in the first experiment (chapter 2).

Maternal effects and their relation to paternal attractiveness

In the two studies described above (chapter 2 & 3) I found that firstly, red junglefowl hens showed positive differential allocation when mated with attractive roosters, as they started to produce a clutch earlier when mated with large-comb males (chapter 2; Lelono et al., in prep a). But I also found evidence for negative differential allocation, as the hens laid heavier eggs when artificially inseminated with T enriched ejaculates, that mimic the ejaculates of unattractive males (chapter 3; Lelono et al., in prep b). The production of heavier eggs was contrary to my hypothesis, (based on a meta-analysis by (Horváthová et al., 2012)), as I expected that attractive males in female-care only species, would induce hens to produce larger eggs. In this study I found no differences in other reproductive variables, such as: the number of eggs or clutch size, the onset of clutch production, or total egg mass, in relation to male attractiveness. How T in the ejaculates affect egg mass is as yet unknown. In fruit flies and birds, the oviduct and storage tubules are innervated by the central nervous system (Freedman et al., 2001; Rodríguez-Valentín et al., 2006). This seems likely to be the case in birds as well. But, how females perceive T in the ejaculate is as yet not entirely clear. This may however be achieved via the presence of androgen receptors in the outer membrane of a hen's reproductive organs (Kawashima et al., 1999; Xunguang et al., 2018; Yoshimura et al., 1993). These receptors could play a role as a sense organ to recognize the presence of androgens, which may then be communicated to the central nervous system which in turn may affect egg production.

As enriched ejaculates mimic the ejaculate of subordinate unattractive males, my result is in line with a study in zebra finches (*Taeniopygia guttata*) (Bolund et al., 2009) and in mallards (*Anas Platyrinchus*) (Bluhm and Gowaty, 2004), in which it was shown that females laid larger eggs when paired with sexually unattractive males. Larger eggs tend to have positive effects on offspring survival and growth (Christians, 2002; Krist, 2011), which indicates that a maternal compensation strategy was present.

Secondly, I found that sons sired by attractive males were growing slower during early development compared to sons of unattractive males, where the opposite was the case for daughters (chapter 3). This difference in growth pattern can be explained by differential sex-specific embryonic exposure to androgens, such as T, that stimulate growth during early development (Groothuis et al., 2005b; Müller et al., 2010). I therefore concentrated on sex-specific yolk androgen deposition, since it

is known that females may differentially deposit these hormones in their yolks based on mate quality reviewed in (Von Engelhardt and Groothuis, 2011). Moreover these hormones are well known to affect offspring growth and behavioural development (Groothuis et al., 2005a; Von Engelhardt and Groothuis, 2011). Yolk T concentrations may be higher after insemination with ejaculate containing elevated levels of T. This is because ejaculate T (being lipophilic), might be entering the yolk in the female tract during fertilization, occurring before the addition of albumin and shell. However, contrary to these expectations I did not find any differential effect of the ejaculate treatment on yolk T or yolk androstenedione concentration. However, it might still be possible that mothers deposit the hormone differently over the sex of her offspring depending on male quality. Unfortunately, in the previous two studies, I did not measure hormone deposition in relation to embryonic sex. Therefore, the main purpose of the next experiment was to investigate whether red junglefowl hens differentially adjusted their yolk androgen deposition according to their partner's social status and embryonic sex. I expected that females adjusted yolk androgen deposition based on both partner quality and the sex of the embryo in such a way that daughters of males with a high social status (winners) and sons of males with a low social status (losers) developed in eggs with higher androgen levels than their siblings of the opposite sex. This hypothesis is based on the sex-specific growth patterns found in our first study although it is opposite classical sex-allocation theory.

I found male status did affect maternal yolk hormone concentration in an embryo sex-dependent way. Androstenedione was significantly higher in eggs containing sons of females paired with losers compared to females paired with winners, whereas the opposite pattern occurred in eggs containing daughters. Although T showed a similar pattern this was not significant (chapter 4). How this sex-specific allocation comes about is as yet unresolved. In birds the female is the heterogametic sex, and thereby potentially have control over the sex of their offspring. However, in the domesticated layer hens, I found the opposite pattern where females allocate more T containing sons of attractive partners compared to unattractive ones (see box 1). Although there are clear differences between the studies (wild versus domesticated chickens, artificially selected for immunity or not) the difference is as yet difficult to explain. In any case it demonstrates that chicken mothers are able to allocate androgens to their eggs in a sex-specific way. As I did not find an overall sex-effect, but only in relation to mate quality, the underlying mechanism must be complex

perhaps interacting with other yolk components that are affected by male quality or other environmental and social factors. As steroid hormones are deposited in the yolk before ovulation and fertilization, either the yolk hormone concentration determines subsequent meiosis and thereby the sex of the egg, or additional hormone is added after fertilization during the addition of albumin, after which the lipophilic steroids move to the yolk. A third possibility is that both the hormone and the sex of the offspring varies with laying order as found by Badyaev et al (2005) but I could not find evidence for this in our study.

A completely alternative mechanistic explanation for the sex-specific yolk hormone concentrations is that these are not due to maternal allocation, but to sex-specific hormone metabolism by the embryo during the first 3 days of incubation. Indeed, avian embryos heavily metabolize maternal androgens already very early in development (Paitz et al. 2011, Paitz and Casto 2012), Kumar et al. 2018a, b) but whether there are sex differences in this respect is currently unknown. However, this alone cannot explain the pattern I found, unless there is also an unknown factor that relates to mate quality that influences these metabolic processes in the embryo. It is conceivable that mate quality affects another component of the yolk that is then perceived by the embryo that in turn affects its hormone metabolism.

Based on sex-allocation theory, as explained in box 1, one would expect that mothers favour investment in sons over daughters when paired with an attractive male in a system like that of the red junglefowl (Clutton-Brock and Parker 1992). In such a system, the variation in reproductive success is much larger in males than in females, and the fitness return of sons of good-quality fathers would be higher than that of daughters. However, our results of the red junglefowl study suggest the opposite pattern in which daughters but not sons are favoured when sired by attractive males. One possible explanation for this is that prenatal T exposure in daughters makes them more attractive to males while sons sired by good-quality males do not need such a boost. Females with larger ornaments (so more male-like) are more attractive to roosters because it signals fecundity (Cornwallis and Birkhead 2007) and yolk T has been demonstrated to promote male-like quality in female comb in this species in terms of coloration (Riedstra et al. 2013). So yolk T might make females more attractive to males. Moreover, egg mass is positively related to female body mass so that allocating more T to female eggs enhances the attractiveness and fitness of the daughters by its effect on growth. In males, the effect of prenatal exposure to T on comb colour was much less than in females (Riedstra et al. 2013). As comb size

Box 1 | Sex-specific yolk androgen allocation related to paternal immunocompetence

We found the sex-specific yolk androgen allocation phenomenon in the highly domesticated Leghorn as well, but in an opposite pattern as in the red junglefowl. In the laying hens we found that mothers deposit more T in sons relative to daughters when mated to males selected for high immunity line, and the other way around when mated with males of lower immunity line. The intriguing question is why mothers differentially allocate androgens to sons and daughters depending on the quality of their partner. Androgens may have beneficial effects on early growth. However, early exposure to androgens may also have negative consequences such as immune suppression, increased metabolic rate and oxidative stress (Duffy et al., 2000; Folstad and Karter, 1992; Gil et al., 1999; Groothuis et al., 2005a; Müller et al., 2005; Owen-Ashley et al., 2004). The pattern of elevated androgen deposition in eggs containing sons when mated with males of good immunity is in line with the hypothesis coined by Gil et al. (1999), in which the only offspring from good-quality parents can bear the cost (decreased immunocompetence) of increased early androgen exposure. The pattern in daughters is however reversed. One possible explanation is that hens mated with high-quality males invest more in sons because the potential fitness rewards of a good-quality son (which can bear the cost of exposure to androgens) are higher than that of a daughter in harem systems like in the red junglefowl, whereas when paired with a low-quality males fitness return of daughters would be higher. However, it is unclear why in the latter case daughters may be able to withstand the costs of T exposure perhaps the T dependent cost-benefit relation differs between the sexes. Indeed, there is ample evidence that yolk T has sex-dependent effects (Riedstra et al. 2013, Tschirren et al. 2015).

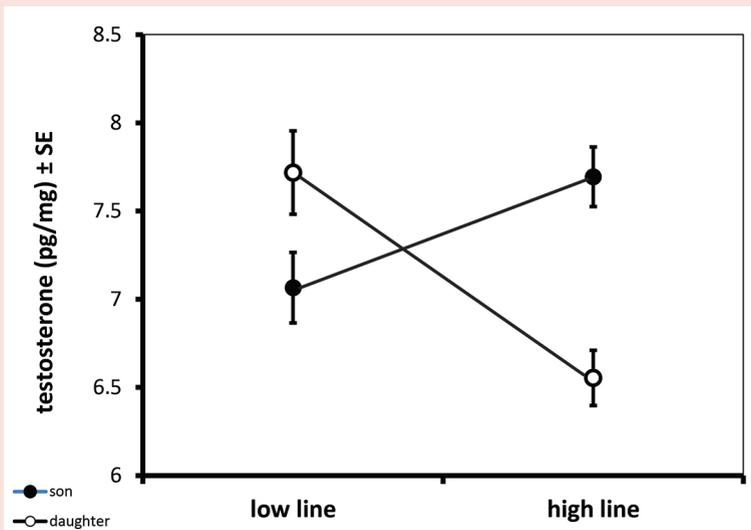


Figure 1 | Mean (\pm SE) in pg/mg yolk T concentrations in eggs containing sons (filled circles) and daughters (open circles) of females

is heritable anyway (Parker 2003) perhaps sons do not need a boost when sired by good-quality father (concerning comb characteristics), but do need it when sired by a poor-quality father. However, this does not explain why daughters would not always receive elevated levels of maternal androgens unless total clutch androgen deposition is limited. This needs further study.

In this study, I found that females allocated different amounts of androgens to eggs containing sons or daughters, depending on the attractiveness of the mate (chapter 4). Sons of attractive males were exposed less to androgens, which then grew slower, than sons of unattractive roosters and the opposite was the case for eggs containing daughters, thereby explaining the observed differences in growth pattern in the chapters 2 and 3.

An important underlying assumption for why mothers differentially allocate T to their eggs in relation to paternal quality is that only offspring of good-quality males can withstand the costs of elevated T exposure (see box 1). However, this has never been tested. I therefore mated white leghorn females of a control line with males from an up and downward selection line for natural antibody production (NAb) (Parmentier et al., 2004; Van Der Klein et al., 2015) (chapter 5), and measured the naturally occurring androgen levels in freshly laid eggs. I then manipulated yolk T levels by injecting T in eggs of both groups of hens. I subsequently examined the effects of the interaction of line and treatment on chick growth, behaviour, and cell-mediated and humoral immunity. I expected that only offspring sired by good-quality males could withstand the costs of maternal androgen deposition to their immune system. However, there were no effects of *in ovo* increased T exposure on cell-mediated or humoral immunity during the first eight weeks after hatching, nor on growth, nor sex-dependent effects in these. There are several possible explanations for our 'negative' results: Firstly, mothers compensated for low parental quality by adjusting other components of the eggs such as depositing immune enhancing factors like carotenoids. Secondly, the effects of embryonic exposure may have only long-lasting consequences whereas I only followed the offspring for the first 8 weeks. The immune system of chicks in the period that I challenged them was still developing (Apanius 1998) and strongly under the influence of maternal immune factors. Thirdly, the elevated prenatal exposure to yolk T may have had effects in domains I did not test, for example, it has been found that yolk T increases metabolic rate (Brown et al 2004, Tobler et al 2007). However, the most parsimonious explanation might be that the hypothesis under testing needs to be rejected. If so, alternative hypotheses

for mate dependent hormone deposition in the yolk should be thought of I could postulate an assumption that T enhances begging and stimulates parental care and mothers do not wish to invest so much in offspring of low-quality.

Testosterone and male reproduction strategies

In group-living animals, social interactions play an important role in determining mating opportunities (Wilson et al., 2009). Socially dominant individuals often monopolize copulations by preventing access of competitors to the female so that subordinates achieve a limited degree of reproductive success (Double and Cockburn, 2003; Pizzari et al., 2007). As subordinate males often constitute the population majority, it is important to understand their alternative reproduction strategies (Double and Cockburn, 2003; Young et al., 2007). When females copulate with multiple males, paternity is determined by the competitive capacity of a male to access females and by the ability of its ejaculates to out-compete those of other males. Although it seems difficult for males to stop females ejecting ejaculates and thereby reducing their chance of siring offspring, there are several males strategies to enhance paternity share: increase T level in the circulation and become more dominant and so acquire matings, modify T levels in the ejaculate if it would amplify sperm motility or produce more ejaculate and sperm.

T is produced by Leydig cell in the testicles, and transported throughout the body via the blood circulation (Deviche et al., 2006; Wingfield et al., 2000). Birds produce variable amounts of T in relation to age, season and other environmental stimuli (Bacon et al., 2000; Rosenstrauch et al., 1998; Wingfield, 1985). In our study (chapter 4) at the group level, the data suggest a trade-off mechanism between T transferred to the circulation and the ejaculate, since winner males increase plasma T while decreasing ejaculate T, (chapter 4). However, on the individual level, data show a lack of correlation between the change in the circulation and the change in the ejaculate. It seems therefore that the regulation of T in the blood circulation and the seminal vesicles is independently regulated. Moreover, in our experiment, winner males' plasma T increased up to 70% directly after a social challenge whereas their decrease in ejaculate T was 40.5%. Given the large difference between the volume of blood plasma in the circulation and the ejaculate volume, the total change in plasma T is larger than that in ejaculate T.

Box 2 | Sperm motility and the possible relationship with T

I found that a fight between large and small-comb males resulted in different levels of T in both the circulation and ejaculates of these males. Loser males had lower T in their plasma but higher in the ejaculate compared to the winner males (chapter 3 and 4). During a copulation, males contribute not only spermatozoa but also hormones and other substances in the ejaculate that may support fertilization success (Anderson and Navara, 2011). The ejaculates are thought to provide optimal conditions for fertilization and contain immunosuppressive substances that protect spermatozoa from damage in the reproductive tract (Pohanka et al., 2002). In insects, the ejaculate substances can exert diverse behavioural and physiological effects in females, including altered longevity and reproductive output (South and Lewis, 2011). I studied whether in red junglefowl, ejaculate composition could be different between dominant and subordinate males and used as a tool to influence female reproductive investment in her offspring which has so far not been addressed in the literature. (chapter 3).

The function of T ejaculate on sperm cells is not yet fully understood. The study conducted by Warikoo et al., (1986) on the bonnet monkey (*Macaca radiata*) showed that motility is directly related to the distribution of T binding sites on the surface of sperm cells. In a recent study, it was found that the presence of androgen receptors was concentrated in the central part of sperm. This section is important for sperm motility because of the presence of mitochondria as an energy source for flagella movement (Naz and Sellamuthu, 2006; Solakidi et al., 2005). This suggests that the function of T in the ejaculate is to increase sperm motility. However, Sexton (1974) found that exposure to T suppresses sperm metabolism that may decrease sperm metabolism but enhances their longevity. Therefore, in a recent study, I analysed sperm motility in the ejaculate of dominant and subordinate males.

I found that red junglefowl loser sperm was more motile than that of the winner's ejaculate (Darybari et al. in preparation). This is in line with our hypothesis, but experimental approaches should confirm this. Higher sperm motility increases the possibility of fertilizing eggs in a competitive situation. (Birkhead et al. 1999; Froman et al. 2002). This suggests that the increasing ejaculate T of the subordinate male is a strategy to compensate their lower mating chances which dominant males may not need.

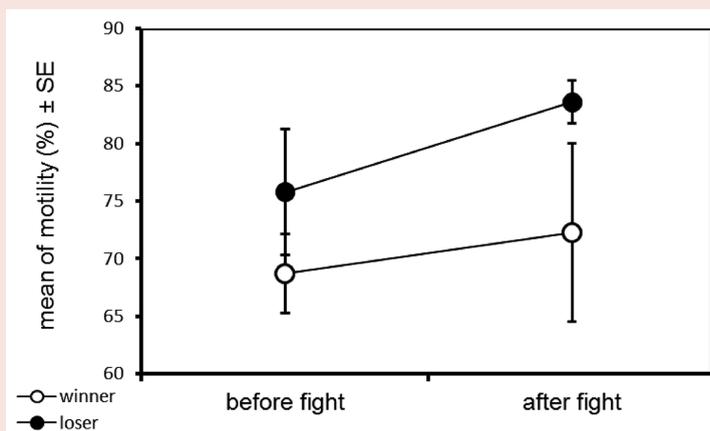


Figure 2 | Sperm motility of loser and winner males. Subordinate males (close dot) possess higher sperm motility than dominant males (open dot) after fighting

To produce such an enormous increase in T in a short time (about 30 minutes), after winning a fight, winners must either be due to a strong increase of T production in the testis themselves, via the pituitary that excretes luteinizing hormone (LH) or to an increase in conversion of A4 in the tissues to T (Balthazart et al., 1980; Cecil, 1983). Of course, these processes could be influenced by many factors such as the quantity of the potential convertible compounds, the availability of enzymes, etc. but it remains unclear why ejaculate T was significantly decreased in winner males when increased ejaculate T levels have the potential beneficial effect of increased egg mass on offspring growth and survival with no obvious direct costs to the male. In mammals, seminal vesicles contain LH receptors, making these male accessory reproductive organs a potential target of direct T regulation by LH (Tao et al., 1998). This presence of LH indicates the seminal regulate T in the ejaculate, facilitating independent regulation of the hormone in the ejaculate and the circulation.

Those mechanistic and functional explanations leave the question as to why winner males decrease their T level in the ejaculate? Does it relate to the physiological cost of produced more T or the conversion of T to its metabolites in the seminal fluid? Although it is well known that T is essential for sperm production, the correlation between ejaculate T concentration and sperm quality remains surprisingly unclear. However, some literature indirectly supports our hypothesis that elevated ejaculate T enhances sperm quality as some studies show that large-comb males have lower sperm quality (Birkhead et al., 1999; Froman et al., 2002).

Similarly, dominant males usually possess larger comb but their sperm is less mobile than that of subordinate males (Navara et al., 2012; Parker, 2006). Pizzari et al., (2007) showed that winner males drop their sperm quality following a social challenge which remained lower for 14 days. Pizzari (2017) suggests that the cost of producing high-quality ejaculates is so high that males are unable to achieve social dominance and make high-quality ejaculates at the same time. However, what exactly the cost is, currently not known.

The role of differences in ejaculate fluid to enhance sperm motility has been studied by Bartlett et al., (2017). They manipulated sperm in vitro combined with competition experiments in the chinook salmon (*Oncorhynchus tshawytscha*). They incubated dominant male sperm in the plasma of subordinates and vice versa. They concluded that subordinate ejaculate plasma significantly enhanced sperm motility. In avian species, high motility gives males an advantage since it enhances the sperm competitiveness to fertilize eggs. Another advantage of high sperm motility

is the higher chance to occupy the sperm storage tubule in the female (SST). This occupation is especially relevant for subordinate males as they have only limited access to females. (Birkhead et al., 1999).

Immuno-competence and the relation to early T exposure

In this study, I tested whether the genetically based immunity of the father determined offspring vulnerability to the potential negative effects of yolk androgens on the immunocompetence of offspring. Our hypothesis is that only offspring sired by good-quality males could withstand the costs of maternal androgen deposition to their immune system. This expectation has been proposed to explain why avian females adjust androgen concentrations in their eggs according to the attractiveness of her mate (e.g. (Gil et al., 1999). In the experiment, I found that egg and yolk mass of females which were forced to mate with low-line males were significantly heavier than when mated with males of the high-line. This indicates that the females were able to perceive quality differences between the males. However, no effects of paternal immunocompetence on yolk hormone concentration was found.

Moreover, in the experiment where I, in a full two by two design, injected eggs of both pairs I did not find any effect of treatment or male quality or their interaction on hatching success, differences in body mass, initial levels of naturally occurring antibodies, or on the immunological responses to three different challenges. I conclude that there was no clear indication that chicks of the high-line were able to handle the negative impact of increased maternal androgen exposure better than chicks from the low-line. However, the immune system of chicks in the period that I challenged them is still developing (Apanius, 1998), in which case the effects of embryonic T exposure may have been only small and difficult to detect. Nevertheless, these results do therefore not support the hypothesis that offspring from low-quality fathers are more sensitive to T exposure during embryonic development than offspring from high-quality fathers. There may be several reasons why I did not find this: Firstly, in contrast to my other experiment with this species, the naive virgin females did not have visual access to other males than their mate. This lack of information could have prevented them from acquiring a reference for the relative attractiveness of her mate. Secondly, an important signal of quality (comb size) (Johnsen and Zuk, 1995; Ligon and Merola-Zwartjes, 1998; Parker, 2003; Parker and Ligon, 2002; Zuk et al., 1990a, 1990b) was lacking due to the practice of comb culling before the roosters arrived in Groningen.

Conclusion and future perspectives

In this thesis, I presented 3 important new findings 1) males have ejaculate T levels (a cryptic paternal trait) that vary systematically with social status and affect female reproductive decisions, 2) Females allocate androgens to the eggs in relation to male quality and offspring sex, and 3) both 1 and 2 lead to differences in offspring development.

Here, I present a schematic of the effect of paternal traits on the maternal reproductive investment. During the mating season, social dyadic challenges induce differential male T allocation to their circulation and ejaculate which in turn affects male attractiveness sperm motility and social status. Those male traits affect female investment (positive differential allocation or negative differential allocation /compensation). More in detail (Figure 3): a male-male interaction results in being either dominant or subordinate, that in turn affects T concentrations in the ejaculate (lower in dominant and higher in subordinate males) and its circulation in the rest of the body (in the opposite manner) (chapter 3 and 4). Dominance may be signalled to the female both directly when she watches the fight (chapter 4) and indirectly by comb size (larger in prospective winners, chapter 2). Circulating T affects in turn comb size as well as fighting success and both increase male attractiveness for the other sex (references in the chapters 2 and 4). Perception of the visual signal comb size leads to faster clutch production (chapter 2) and perception of social status (and perhaps comb size) leads to sex-specific T allocation in eggs (more in male eggs than female eggs when mated to a small-comb male: chapter 4) and the other way around when fertilized by T enriched ejaculate (chapter 3). This is reflected in sex-specific growth patterns of the chicks (chapter 2). However, in contrast to the expectation, differential yolk androgen hormone deposition did not affect offspring immunocompetence (chapter 5). Therefore, the hypothesis, posed in the literature, that avian mothers deposit lower levels of androgens in their egg when mated with an unattractive male since due to its lower immunocompetence the chicks would not be able to withstand the costs of T exposure, could not be supported, even when I used males that differ in immunocompetence.

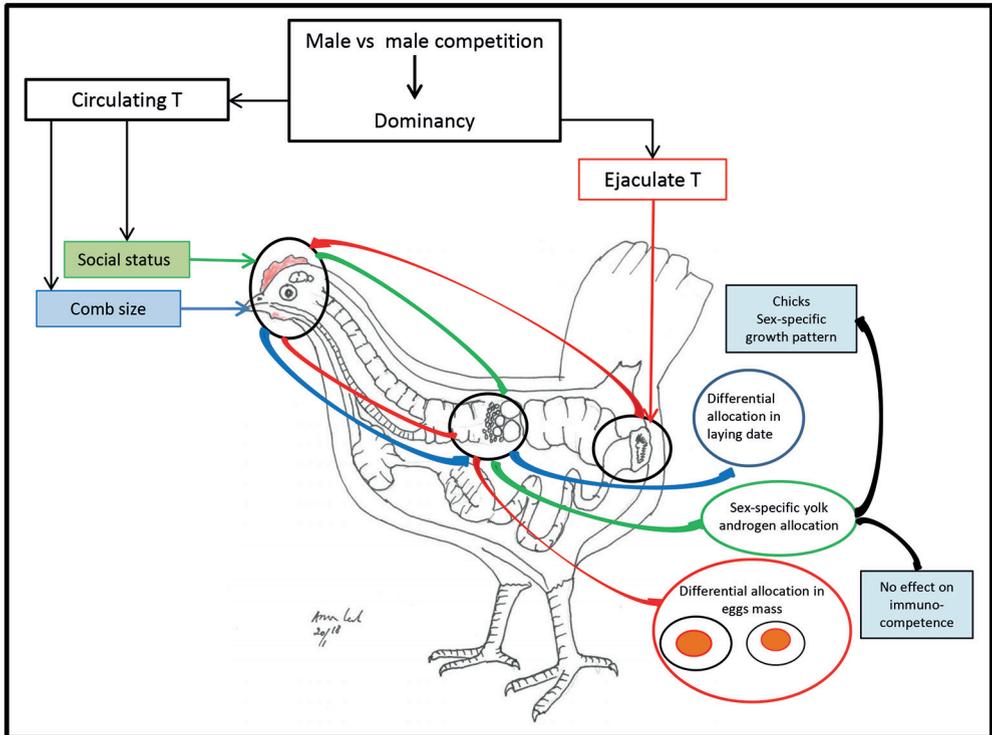


Figure 3 | The effects of paternal traits on maternal effects. The colours show the pathway of the different paternal traits on the female and her offspring

Several questions that now need to be addressed are largely in the domain of physiology: How do males regulate T levels in different parts of their body, how (if at all) does it affect sperm quality, and how does ejaculate T translate to its effect on the female and the chick? There are also questions remaining about female reproduction: how do females differentially allocate androgens to eggs containing either sons or daughters and in which context do they apply this and in what way? Furthermore, It remains unknown to what extent and in what domain chicks suffer (or benefit) from androgen exposure, and what is the effect on maternal and chick fitness, since why else would females (sex-specifically) systematically deposit different amounts of androgens in their eggs. Moreover, can embryos have different strategies to deal with this exposure in case of parent offspring conflict? These questions are interesting because parental effects have already been intensively studied for several decades but with the focus on maternal effects lacking the family context in which males and offspring may play their own role (Groothuis et al., 2019). A particularly

promising subject for future research is the impact of social status on the dynamics of circulating and ejaculate androgen levels because these levels affect the male, the female, and the offspring. In conclusion, I have strengthened and opened new pathways for a better understanding of the often reported differences in maternal effects by studying them in the context of male-female interactions.

