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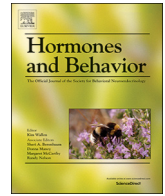
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The relationship between male social status, ejaculate and circulating testosterone concentration and female yolk androgen transfer in red junglefowl (*Gallus gallus*)

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ABSTRACT

Several studies show that avian females prefer males based on their secondary sexual ornaments and dominance status. We tested in red junglefowl (*Gallus gallus*) how comb size affected the result of fighting and how the dominance status related to testosterone concentrations in their circulation and ejaculates. We subsequently tested how social status was related to female reproductive investment, including yolk hormone transfer. We found that after a fight 1) winners increased plasma T and decreased ejaculates T whereas losers' T remained unchanged, and 2) plasma T of winners was higher but ejaculates T was lower than those of losers. We argued those are consistent with the different reproductive strategies of dominant and subordinate males. Furthermore, in line with offspring sex-dependent growth patterns females transferred significantly more androstenedione to female than male embryos when mated with winners, while doing the opposite when mated with losers. We concluded therefore that female reproductive investment was affected by both partner quality and embryo sex. The results indicate that male quality influences sex-specific maternal investment, which could be mediated by ejaculate testosterone concentration.

1. Introduction

Life history theory predicts that parents should adjust their investment in current reproductive attempts by weighing the relative benefits against future reproductive attempts (Trivers, 1972). One influence on this trade-off is the quality of their mate (Alonso-Alvarez and Velando, 2012). Male secondary sexual characters might be expressed in large antlers (Vanpé et al., 2007), bright colours (Osorio and Vorobyev, 2008), and large song repertoire (Pfaff et al., 2007). Such characters influence female mate choice (Andersson and Iwasa, 1996). This preference can be advantageous when these male characters are either heritable, making their offspring sexually attractive, and/or somehow honestly reflect male quality, e.g., parental investment, age, health, or social status (Parker and Ligon, 2002). In line with this study, female birds generally invest more in offspring when paired with preferred than with un-preferred males (Horvátová et al., 2012). This is known as the (positive) differential allocation hypothesis (DAH) (Burley, 1986; Ratikainen and Kokko, 2010; Sheldon, 2000) and has been shown for several reproductive variables, such as offspring provisioning (Pryke and Griffith, 2010), androgen deposition in the egg (Gil et al., 1999), egg mass (Cunningham and Russell, 2000; Soma and Okanoya, 2013),

or clutch size (Loyau et al., 2007). The result showed that offspring quality may be better as has been shown by positive correlations between paternal attractiveness and offspring quality (Petrie and Williams, 1993; Sheldon et al., 1997). Females paired up with less attractive males, however, can also be expected to invest more in the current reproductive attempt to compensate for the negative effects of low-quality fathers (reproductive compensation hypothesis; Gowaty et al., 2007; Ratikainen and Kokko, 2010; Kindsvater and Alonzo, 2014), as was shown in mallards *Anas platyrhynchos* (Bluhm and Gowaty, 2004; Gowaty, 2008).

The effects that males exert on maternal investment, whether positive or negative, is an intriguing example of an interaction between a paternal trait and a maternal effect. Maternal effects are known to profoundly influence development, especially during the early stage when the embryo is very sensitive to environmental cues and they can have long-lasting effects on the offspring (Badyaev and Uller, 2009; Groothuis et al., 2005a; Mousseau and Fox, 1998). This form of non-genetic or indirect genetic effect mediated by parents is increasingly recognized as being important in understanding heritability, adaptation, and evolution.

The sexually dimorphic red junglefowl (*Gallus gallus*) is a well-

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Table 1

The scheme of result and prediction. In black summary of main findings of our previous work and in red hypothesis derived from this relevant for this paper. See text of the Introduction.

	Large-comb males	Small-comb males
Before fight		Circulating and ejaculate T similar?
After fight	Circulating T high and Ejaculate T low Son grow faster than daughter Yolk T higher in eggs containing sons than daughters?	Circulating T low and Ejaculate T high Sons grow slower than daughters Yolk T lower in eggs containing sons than daughters?

known study species for the investigation of mate choice and maternal effects. Comb size of males in this species is an essential signal for female mate choice (Parker and Ligon, 2003). Comb size is related to health (Zuk and Johnsen, 2000; Zuk et al., 1990a, 1990b), dominance status (Johnsen et al., 2001; Ligon et al., 1990; Zuk and Johnsen, 2000), and circulating (plasma) testosterone levels (Parker et al., 2002). Comb size is heritable (Parker, 2003) and is also correlated with heritable variation in body condition (Parker and Ligon, 2007). This heritability is advantageous for females to choose large-comb males as fathers for their offspring (Ligon and Merola-Zwartjes, 1998; Parker and Ligon, 2003).

The current study is based on our previous studies summarized in Table 1. In one previous study, (Lelono et al., 2019), we found that large-comb males are always winners in a confrontation with small-comb males and after the social challenge have higher plasma T and lower ejaculates T compared to the subordinate males. However, whether these differences already existed before fight, for example related to the social status or physical characters such as body mass and comb size, or are due to winning or losing a fight remains to be tested. We also found that small-comb males produced slow growing daughters and fast-growing sons, and large-comb males produced fast-growing daughters and slow-growing sons (Lelono et al., 2019b). We also found that the testosterone enrichment in the ejaculates of dominant males with low concentrations of T, mimicking the higher T concentrations in the ejaculates of subordinate males, resulted again in sons from T-enriched ejaculates grew faster and daughters grew slower than offspring from control ejaculates, consistent with our previous study. This sex-specific growth pattern may be explained by differential sex-dependent androgen transfer by the mother to her eggs since androgens have been shown to affect chick growth (Groothuis et al., 2005a, 2005b; Müller et al., 2005; Von Engelhardt and Groothuis, 2011), and can be differentially transferred to the egg depending on both environmental factors and sex of the embryo (Badyaev, 2005; Müller et al., 2002; Pariser et al., 2012; Rutstein et al., 2005). However, also this remains to be tested as we did not measure hormone concentration in relation to embryonic sex in the previous experiments.

The main aim of this paper is to understand the relation between partner quality and androgen sex-specific transfer to the egg. Therefore, we investigate how males adjust their T in blood circulation and ejaculates after a dyadic social challenge and how it impacts on the female androgen hormone transfer in relation to offspring sex. We determined ejaculates and plasma T in roosters before and just after a social challenge and to disentangle whether the outcome of a fight or the inherent characters of the males were responsible for the differences in ejaculates T. We then paired up hens either with a male that had won or had lost a fight in the presence of the female. Hens were subsequently allowed to produce a clutch. After clutch completion, we repeated the experiment but reversed partners (from winner to loser and loser to winner). In both reproductive attempts, we recorded egg mass, clutch size and the latency to produce the first egg. Yolk androgen concentration was measured after 3 days of incubation, when the sex of the embryos could be determined. We expected that males that won the challenge had higher plasma T and lower ejaculates T than roosters that lost. Furthermore, we expected that females adjusted yolk androgen transfer according to both partner quality and the sex of the embryo in

such a way that daughters of winners and sons of losers developed in eggs with higher androgen levels than their siblings of the opposite sex (see Table 1).

2. Methods

2.1. Animals

We used 24 male and 24 female captive red junglefowl (*Gallus gallus*) from our facility (the University of Groningen, The Netherlands), which is an outbred population of wild-caught birds originally from Asia. All males display the species-specific calls, much shorter than those from domestic chicken, and most junglefowl still moulted their neck feathers in autumn. Before the experiment, all male and female fowl were housed in large and roofed aviaries in sex-specific flocks, with ad libitum access to food, water, grit, perches, and dustbathing substrate. During the experiment they were housed individually, with visual contact to conspecifics in identical aviaries (1 × w × h: 1.5 × 4 × 2 m) with the same facilities and nesting possibilities. Both the potential winners and losers were previously used in an experiment where we investigated the effects of testosterone in the ejaculates on female reproductive decisions (Lelono et al., 2019).

2.2. Experimental design

At first day of the experiment, 24 males consisted of 12 large comb and heavy males (potential winner/W) and 12 small comb and lighter males (potential loser/L) in individual cages. The individual cages were made in which all the cages were connected by wire mesh to avoid social isolation. On day 8 we obtained a blood and ejaculates sample of all males (baseline sample).

Based on biometrical measurements taken at the beginning of the experiment, we selected and matched pairs in such a way males to allows easy prediction on which one will become dominant during an agonistic encounter. Then on day 15, these pairs of males were placed together in the middle of a fighting arena, with a single female in each side of the adjacent aviary at each side of the male compartment, separated by wire so that both females could observe the fight (see Fig. 1). Males were left in this condition for 15 min, during which they fought over dominance. The winner and loser were then determined for each pair and all large-comb males won the agonistic encounters. After that, the males were housed individually in one of 24 single aviaries that were now visually isolated from each other and left to recover for 30 min after which a blood and ejaculate sample were taken. Then the two females present during the fight were randomly housed either with the winner or the loser of that confrontation and given the opportunity to mate and produce a clutch.

At clutch completion, males were moved to their individual aviaries. After one week of recuperation, the procedure of the dyadic confrontations was repeated with the original females present in the side aviaries. All initial winners were also the winners in the second dyadic encounter. Subsequently, females were assigned to the other male of the winner-loser pair than in the first batch and allowed to produce a second clutch. From both clutches, clutch size, egg mass, clutch initiation time, embryo sex and yolk hormone concentration

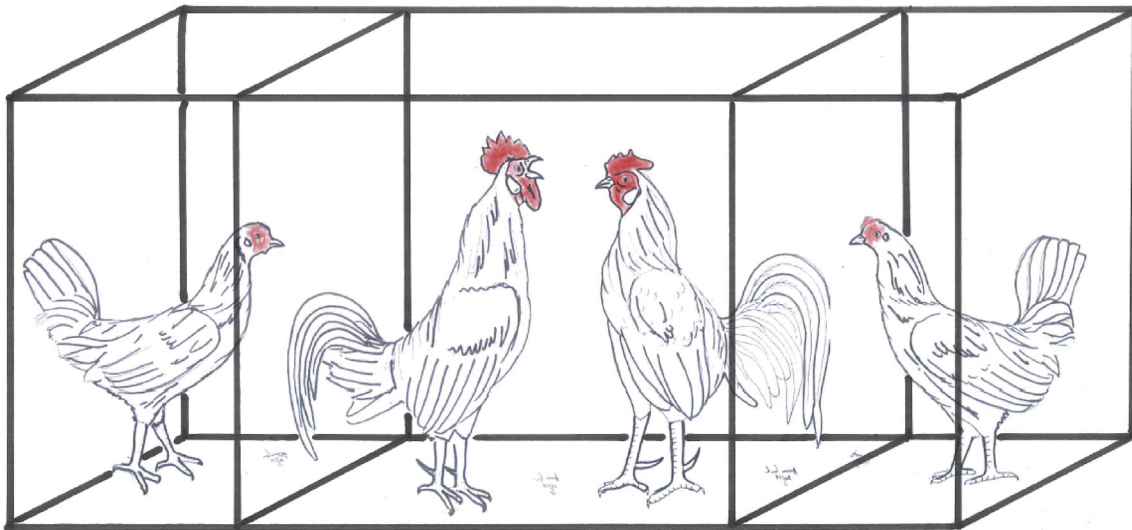


Fig. 1. The fighting arena. Two males were housed in the center of the arena and both females were housed in the adjacent cages. Wire mesh was fitted between the center of the arena and the adjacent females cages to allow the females to observe the males.

(testosterone (T) and androstenedione (A4)) were determined.

2.3. Biometry

We determined body mass, tarsus length, spur length and comb size (surface area) of all males at the beginning of the experiment to select roosters that were most likely to win agonistic interactions and become dominant. Body mass was measured to the nearest gram. Tarsus length were measured in mm (one digit) and spurs length were measured in mm (one digit). Rooster combs were photographed using a digital camera (Canon SX 500 IS) with a round sticker ($d = 0.5$ cm) attached as a reference. Pictures were taken only from the left side of the rooster heads. The number of pixels in the photographs of the combs were counted using GIMP 4.8 and calibrated against the number of pixels of the sticker with known surface area. The twelve males with the largest combs were assigned to the large-comb group, and the remaining to the small-comb group. All pre-selected large-comb males won every staged dyadic agonistic encounter and were heavier than the pre-selected small-comb males both at the beginning of the experiment and just before the second staged agonistic encounter (see Table 1). Body mass of the females was also measured just before the onset of the experiment and after completion of the first clutch. Females were balanced across the 2 treatments in such a way that the average body mass of both groups was similar (see Table 2, first row).

2.4. Blood and ejaculates sampling

On day 8 (baseline sample in isolation before the experiment) and 15 (the day after the first dyadic encounter), we collected a blood sample and an ejaculates sample of all males. We collected about 1 ml

of blood from the ulnar vein within 3 min (after opening the door of the aviary and handling the rooster) using a syringe with a 25G needle. The tube of blood sample was pre-rinsed with EDTA (9 g/100 ml) to prevent coagulation. Samples were then centrifuged for 10 min at 2000g, and circa 0.5 ml of plasma was then separated and stored at -20°C until analyses.

Ejaculates samples were collected after all roosters were first trained using the abdominal massage method (Burrows and Quinn, 1939; Parker and Ligon, 2003) to produce ejaculates to a handler. In short, the cloaca of a rooster was massaged with the thumb on one side and the other four fingers on the other side while the back and tails were gently stroked with the other hand. After about half a minute the ejaculates was released and caught in a tube. Ejaculates samples (ca. 0.4 ml) were stored at -20°C prior to analyses.

2.5. Plasma and ejaculates hormone assays

Plasma and ejaculates concentrations of testosterone T were quantified by radioimmunoassay. To extract the hormones, 407 mg of plasma and 368 mg of ejaculates were weighed (accuracy 1 mg), 300 μl of milliQ water was added, and 50 μl of 3H-labeled testosterone (NET553, Perkin Elmer) was added to trace the recovery of extracted hormones. This solution was incubated for 15 min at 37°C before being extracted in 2 ml of diethyl-ether/petroleum-ether (DEE/PE, 70/30 v/v) by vortexing for 60 s. Obtained samples were centrifuged at 2000 rpm for 3 min (4°C) to separate the ether phase, the samples were snap-frozen and the ether/hormone phase decanted into a fresh tube. The extraction procedure was repeated twice with an additional 2 ml of DEE/PB, vortexed for 30 s and 15 s, respectively. Next, the extracts were dried under nitrogen at 37°C . Hormone extracts were rinsed in

Table 2

Independent sample t-test of the comb size and body mass, tarsus and spur length between 12 winner males and 12 loser males at two different time points.

Male status	Before the first clutch					Before the second clutch				
	Winner	Loser	t-Test	p	d	Winner	Loser	t-Test	p	d
	Mean \pm SE	Mean \pm SE				Mean \pm SE	Mean \pm SE			
Comb size (cm^2)	16.6 (0.7)	11.1 (0.3)	8.07	< 0.001	0.86	17.0 (0.7)	14.7 (0.5)	2.69	0.014	0.48
Body mass (g)	1249.2 (21.5)	1039.7 (38.5)	4.75	< 0.001	0.69	1280.0 (28.4)	1072.2 (34.1)	4.68	< 0.001	0.69
Tarsus (mm)	83.2 (0.5)	79.2 (1.2)	3.201	0.005	1.31	83.2 (3.4)	79.2 (1.2)	3.201	0.005	1.31
Spur (mm)	30.4 (0.9)	28.7 (1.0)	1.270	0.217	0.51	31.8 (0.9)	29.9 (1.0)	1.361	0.187	0.56

2 ml of 70% methanol to precipitate any lipids and stored at least overnight at -20°C . Subsequently, the tubes were centrifuged, decanted into a fresh tube, re-dried under nitrogen at 50°C and stored at -20°C .

Before assay, extracts were thawed and dissolved in 125 μl phosphate-buffered-saline with gelatin (PBSG) for plasma and 150 μl PBSG for the ejaculates. Recoveries of the initially added labeled T were measured in a subsample of this solution using scintillation cocktail (Ultima Gold, Perkin Elmer) and radioactivity counted on a liquid scintillation counter. Average recovery was 86% for plasma (SD 4.2%) and 87% for ejaculates (SD 3.5%). Subsequently, 25 μl of the extracted sample was used for [T] determination using a commercial kit (TESTO-CT2, Cisbio Bioassays, Codolet, France). Cross-reactivity was 2.6% and 1.7% for 5α -dihydrotestosterone and androstenedione respectively. Standards were prepared using dilution series from pre-prepared stock and ranged from 0.08 to 20 ng/ml T. Intra-assay for plasma and ejaculates were respectively 2.23% and 2.53%.

2.6. Female reproductive investment

Each morning (regularly between 10 and 12 a.m.) we visited the aviaries to record egg laying. Eggs were collected, weighed, and marked (with a non-poisonous felt-tipped pen for identification purposes) until clutch completion. Eggs were separated into two groups, the odd order eggs were stored at -20°C and the even order eggs were incubated at 37.5°C for three days and then stored at -20°C . All incubated eggs were analysed for embryo sex and yolk androgen concentration. In total, we found 112 embryos (56 males and 56 females) from 24 females. The sex ratio did not systematically vary with laying order (see Results).

2.7. Embryo sexing, and yolk hormone assay

The frozen (-20°C) eggs were thawed at room temperature and the shell and albumen were removed. Then the embryos were collected and conserved in 90% ethanol awaiting molecular sexing. Molecular sex determination of all embryos was carried out by amplification of sex-specific gene sequences (W-chromosome specific primers) after DNA extraction from small tissue samples. All the procedure was performed based on Clinton (1994). When the embryos were removed, the yolks were collected, and their weights were recorded. Yolk T and A4 were then quantified by radioimmunoassay after extraction of hormones. To extract the hormones, 198 mg of yolk/milliQ water mixture (1 + 1) was weighed (accuracy 1 mg), 300 μl of milliQ water was added, and 50 μl of ^3H -labeled testosterone (NET553, Perkin Elmer) was added to trace the recovery of extracted hormones. This solution was incubated for 15 min at 37°C before being extracted in 2 ml of diethyl-ether/petroleum-ether (DEE/PE, 70/30 v/v) by vortexing for 60 s. Extracted samples were centrifuged at 2000 rpm for 3 min (4°C) to separate the ether phase, the samples were snap-frozen and the ether/hormone phase decanted into a fresh tube. The extraction procedure was repeated twice with an additional 2 ml of DEE/PB, vortexed for 30 s and 15 s, respectively. Next, the extracts were dried under nitrogen at 37°C . Hormone extracts were rinsed in 2 ml of 70% methanol to precipitate any lipids and stored at least overnight at -20°C . Subsequently, the tubes were centrifuged, decanted into a fresh tube, re-dried under nitrogen at 50°C and stored at -20°C . Prior, to assay, extracts were thawed and dissolved in 300 μl phosphate-buffered-saline with gelatine. Recoveries of the initially added labeled T were measured in a subsample of this solution using scintillation cocktail (Ultima Gold, Perkin Elmer) and radioactivity counted on a liquid scintillation counter. The average recovery was 89% (SD 2.5%). Subsequently, 25 μl of the extracted sample was used for [T] determination using the same kit as above. Standards were prepared using dilution series from pre-prepared stock and ranged from 0.08 to 20 ng/ml. A4 was determined using 50 μl of the extracted sample (dilution $\times 21$) and a commercial kit (DSL-3800, Beckman Coulter GmbH, Sinsheim, Germany). 'Pools' of yolks

previously repeatedly analysed were used as external controls, and intra assay for [T] and [A4] was 3.74%, and 4.1% respectively.

2.8. Statistical analysis

The differences of comb sizes body mass, tarsus, and spurs between winners and losers were tested by independent *t*-tests. The differences in these traits within winners and losers before and after a fight were compared using paired *t*-tests. The data of testosterone concentration in the plasma and ejaculates within and between winners and losers were \log_{10} -transformed before the statistical analysis to meet the requirements for parametrical testing. The differences of T within and between winners and losers were subsequently compared by using paired and independent *t*-tests. We used Spearman correlations to investigate the association between the change in plasma T and ejaculates T from before and after the encounter in winners and losers.

The effect of male status (winner or loser) on female reproduction was tested using linear mixed model with individual hen as a random effect. The models included clutch order, male status, and the interaction between clutch order and male status as fixed effects.

For yolk T and A4 concentration, we fitted a linear mixed model with each embryo nested in mother as a random effect. The model included male status, sex of the embryo and the interaction between male status and sex of the embryo as fixed effects.

The potential effect of treatment and laying order or their interaction on embryo sex was tested with a binomial generalized mixed model. The dependent variable is the sex of the embryo coded as zero or one. We analyse it using binomial generalized mixed model with laying order nested in mother as a random intercept effect. The model included treatment, laying order of the eggs and the interaction between treatment and order as fixed effect(s).

All analyses were performed using SPSS 23. To determine effect sizes, partial eta-squared (η^2) was calculated for all linear mixed models and Cohen's *d* was calculated for all paired *t*-tests and independent *t*-test.

3. Results

3.1. Male testosterone

Before the encounters plasma and ejaculate T were not significantly different between males that subsequently won or lost ($N = 12$ vs 12 , plasma: $t = -1.23$, $p = 0.24$, $d = 0.55$; ejaculates: $t = -0.85$, $p = 0.44$, $d = 0.25$; Fig. 2). Directly after the encounter, plasma T in the winners significantly increased ($t = 2.82$, $p = 0.02$, $d = 0.82$), whereas their ejaculates T significantly decreased ($t = -2.87$, $p = 0.02$, $d = 0.83$). Plasma and ejaculates T did not significantly change in the losers ($N = 12$, plasma: $t = 0.68$, $p = 0.55$, $d = 0.19$; ejaculates: $t = 0.46$, $p = 0.66$, $d = 0.13$). As expected, after the fight plasma T of winners was significantly higher and ejaculates T significantly lower than those of losers (plasma: $t = -2.29$, $p = 0.04$, $d = 0.66$; ejaculates: $t = 2.39$, $p = 0.04$, $d = 0.69$). There was however no association in either winners or losers between the change in plasma T before and after the encounter and the change in ejaculates T (winners: $r_s = -0.01$, $p = 0.97$; losers $r_s = -0.47$, $p = 0.12$).

3.2. Female reproductive investment, yolk androgen concentration and embryo sex

Clutch initiation time, average egg mass, clutch size, and total clutch mass were not significantly affected by male status nor clutch order, nor by the interaction of these two variables (see Table 3). Male status, however, 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 (see Fig. 3a and Table 4), whereas the

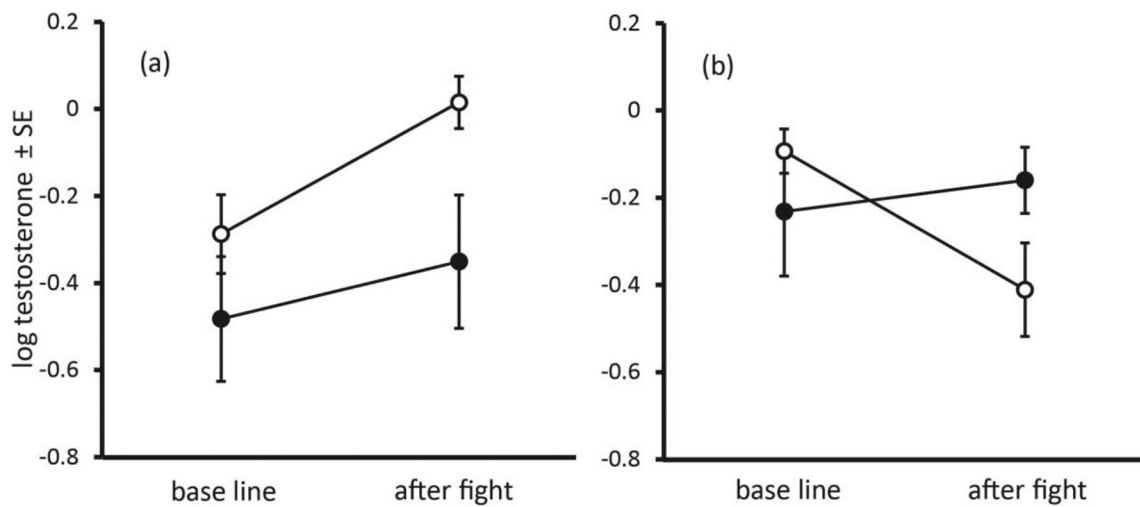


Fig. 2. Mean (\log_{10} transformed data \pm SE) of testosterone concentration (pg/mg) in plasma (panel a) and ejaculates (panel b) of males that won (open circles) or lost (filled circles) a staged dyadic agonistic encounter.

opposite pattern occurred in eggs containing daughters. A similar pattern emerged for T, but it was not significant (Fig. 3b).

There was no effect of the treatment or laying order or their interaction on embryo sex (Wald Chi-Square₍₃₎; $p = 0.991$).

4. Discussion

4.1. Male testosterone dynamics

We analysed the association between male quality and T in males' blood circulation and ejaculates, and their potential effect on female investment, including sex-specific yolk hormone transfer. We showed that there was no difference in plasma and ejaculates T between males prior to an agonistic encounter. But after an encounter, large-comb and heavier males that won the fight (in all cases) had a higher plasma and lower ejaculates T than small-comb and lighter losers (Fig. 2). This result demonstrated that indeed the outcome of the social dyadic challenge instead of physical characters such as body mass and comb size induces the differences in both circulating and ejaculates T between males.

The increase in plasma T by winners is in line with the literature. Parker et al. (2002) found a positive relationship between plasma T, comb size, and dominance rank in male red junglefowl. Furthermore, Ligon et al. (1990) and Johnsen and Zuk (1995) also found that increased T during aggression was associated with winning in an escalated fight. The presence of testosterone in male chicken's ejaculates has been demonstrated (Anderson and Navara, 2011; Zeman et al., 1986). However, to the best of our knowledge it has not been published before that winning a social challenge not only increases circulating T but at the same time decreases T in the ejaculates of an organism. At first glance, this suggests a trade-off between transferring T to the plasma

and the ejaculate. However, since 1) we found no correlation between the change in ejaculates and plasma T before and after a fight and 2) we estimated that the total volume of blood plasma in a rooster was at least 50 fold that of the total ejaculates volume, it was more likely that our result was caused by independent regulation.

The increased plasma T may come from an increased production and allocation of T by the Leydig cells to the blood circulation (Wingfield and Wada, 1989; Wingfield, 1985), whereas the decrease of ejaculates T may be due to the conversion of T to its metabolites, perhaps in the seminal vesicles (Cabeza et al., 2002; Mindnich et al., 2005). This requires further investigation. From a functional perspective this change in allocation after a fight seems adaptive: Dominant males are in the position to monopolize females and increased T in their circulation facilitates this, whereas subordinate males should avoid fights and invest in their ejaculates for sperm competition when given the sparse opportunity to copulate. However, the extent to which T positively affects sperm motility is unclear.

Several studies indicate that dominant roosters produce sperm with lower motility than subordinate roosters (Froman et al., 2002; Graves et al., 1985; Parker et al., 2006). Also, sperm quality (motility in this case) of males that became subordinate after a social challenge was higher than that of males that became dominant (Pizzari et al., 2007). Although the literature is inconclusive about the relationship between sperm quality and ejaculates T (Lelono et al., 2019) the above mentioned studies in combination with our result strongly suggested that ejaculates T indeed positively affected sperm quality.

4.2. Female reproductive investment

Previously we have shown that after artificial insemination with T-enriched ejaculates, mimicking the ejaculates of subordinate males,

Table 3

Reproductive parameters of 24 hens. The effect of male status on females reproductive parameters were tested using linear mixed model with individual hen as a random effect. The models included clutch, male status, and the interaction between clutch and male status as fixed effects.

Male status	first clutch				second clutch				Male status			Clutch			Male status \times clutch		
	Winner		Loser		Loser		Winner		F _(ndf,ddf)	p	η^2	F _(ndf,ddf)	p	η^2	F _(ndf,ddf)	p	η^2
	mean \pm SE	mean \pm SE	mean \pm SE	mean \pm SE	mean \pm SE	mean \pm SE											
Clutch initiation time (day)	7.7 (0.6)	10 (1.4)	6.9 (0.9)	7.2 (0.5)	1.34 _(1,44)	0.273	0.03	3.96 _(1,44)	0.066	0.08	2.06 _(1,44)	0.152	0.04				
Clutch size (g)	8.5 (0.4)	8 (0.7)	7.5 (1.1)	6.9 (0.6)	0.00 _(1,22)	0.949	0.00	2.55 _(1,22)	0.124	0.06	0.51 _(1,22)	0.483	0.02				
Average egg mass (g)	37.5 (0.4)	36.6 (0.7)	38.7 (0.4)	37.6 (0.7)	0.94 _(1,22)	0.344	0.04	0.12 _(1,22)	0.731	0.00	0.12 _(1,22)	0.736	0.00				
Clutch mass (g)	318.9 (15.5)	291.1 (20.0)	300.1 (33.8)	243.3 (20.5)	0.01 _(1,22)	0.927	0.00	4.26 _(1,22)	0.051	0.16	0.95 _(1,22)	0.341	0.04				

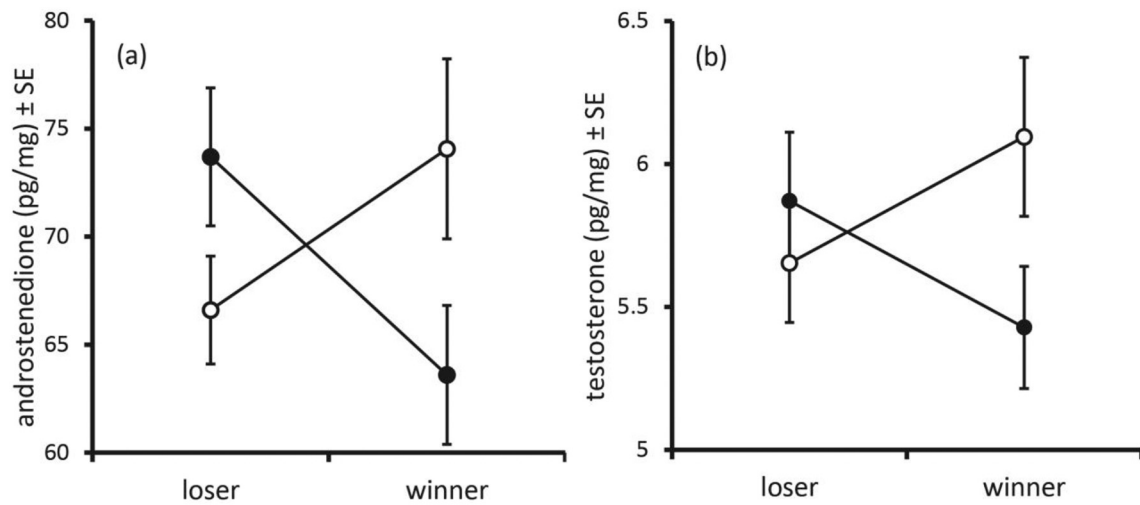


Fig. 3. Mean (± SE in pg/mg) of yolk androstenedione (panel a) and testosterone (panel b) concentrations in eggs containing sons (filled circles) and daughters (open circles) of females that were paired with a male that had won (winner) or lost (loser) a staged dyadic agonistic encounter.

female red junglefowl produced heavier eggs (Lelono et al., 2019). In this experiment, we found no differences in egg mass, clutch initiation time, clutch size, or yolk androgen concentration, between females mated with winners or losers. However, we did find that the yolk androgen concentration in three days incubated eggs were dependent on both male success in a fight and embryo sex.

Females mated with the winner males, transferred more A4 to eggs containing daughters and less to eggs containing sons, and the reverse pattern was the case for females mated with the loser males. A similar although not significant pattern occurred for yolk T. Since androgen exposure may enhance early growth (Von Engelhardt and Groothuis, 2011), this pattern of yolk androgen transfer can explain our previous results that demonstrated the paternal traits induced a sex-specific maternal effect on growth in their offspring. Female chicks grew faster than male chicks when the father had a large comb and slower when the father had small-comb (Lelono et al., 2019b). A similar result was found when we inseminated females with T-enriched ejaculates (mimicking ejaculates of subordinate males) or control ejaculates mimicking that of dominant males (Lelono et al., 2019). This sex-specific T transfer to the egg is contrary to the classical sex allocation theory for social systems in which the variation in male reproductive success is larger than in females (Clutton-Brock, 1989). In our case it seems that females are not investing more in sons when sired by attractive fathers, but rather follow a compensatory strategy. As sons from large comb fathers are known to be of better quality than those of small comb fathers (Parker, 2003) mothers may compensate their daughters for their weaker position in the sibling rivalry after mating with a good quality father. Mothers, paired with poor quality fathers may then compensate their sons for their lower quality. The fact that mate quality can affect yolk hormone transfer of the mother has been repeatedly reported (Garcia-Fernandez et al., 2010; Gil et al., 1999, 2004; Loyau and Lacroix, 2010; von Engelhardt et al., 2006), but other studies did not find this (Cucco et al., 2011; Horváthová et al., 2012; Kingma et al.,

2009). As demonstrated in our results, we did not find an overall effect of mate quality on yolk hormone concentrations, but only in interaction with offspring sex.

Similarly, several studies have analysed overall androgen differences between eggs containing sons and daughters without taking mate quality into account and found no sex differences (Eising et al., 2003; Loyau et al., 2007; Müller et al., 2002; Rubolini et al., 2011). However, similar to us some other studies analysed such sex-specific hormone transfer in relation to a third factor and did find differences in androgen concentration between male and female eggs. For example in relation to maternal dominance in white leghorns, *Gallus gallus domesticus*, (Müller et al., 2002), maternal diet, laying order and mate attractiveness in zebra finches *Taeniopygia guttata* (Rutstein et al., 2005; Gilbert et al., 2005; Pariser et al., 2012), although mate attractiveness did not reach statistical significance. Perhaps sex-specific maternal androgen transfer is much more prevalent than often assumed when taking environmental and social factors into account.

How this sex-specific transfer comes about is as yet unresolved. In birds, the female is the heterogametic sex. As steroid hormones are transferred in the yolk before ovulation and fertilization, either the yolk hormone concentrations affect 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 change over to the yolk. Other possibilities concern hormone-dependent yolk resorption or fertilization in case hormone concentrations do not match the sex of the egg (for a similar discussion regarding sex ratio adjustment in relation to yolk hormones see Goerlich-Jansson et al. (2013)). However, as we did not find an overall sex effect on hormone concentration but only in relation with mate quality, the underlying mechanism must be even more complex. This could imply interactions with other yolk components that are affected by male quality. Badyaev et al. (2005) suggested that sex specific hormone deposition is due to the fact that eggs are clustered according to sex in the laying order and that laying

Table 4

Yolk androgen concentration after 3 days of incubation. The differences of testosterone and androstenedione were tested using linear mixed model with each of the embryos nested in mother as a random effect. The models included male status, sex of the embryo and the interaction between male status and sex of the embryo as fixed effects.

Male status	Winner (mean ± SE)		Loser (mean ± SE)		Male status			Sex of embryo			Male status × sex of embryo		
	Sons	Daughters	Sons	Daughters	F _(ndf,ddf)	p	η ²	F _(ndf,ddf)	p	η ²	F _(ndf,ddf)	p	η ²
Androstenedione	74.4 (4.3)	62.1 (3.2)	67.2 (3.5)	72.3 (2.7)	0.13 _(1,108)	0.716	0.00	0.22 _(1,108)	0.641	0.00	5.39 _(1,108)	0.017	0.05
Testosterone	5.4 (0.3)	6.1 (0.2)	5.9 (0.2)	5.7 (0.2)	0.00 _(1,1038)	0.999	0.00	0.75 _(1,108)	0.387	0.01	2.89 _(1,108)	0.092	0.03

order relates to yolk hormone deposition. This was not the case in our study as the sex ratio did not differ over the laying sequence in either of the experimental groups. Interestingly, females are known to have steroid receptors in their oviduct (Kawashima et al., 1999; Xunguang et al., 2018; Yoshimura et al., 1993) via which male ejaculate T may affect female egg provisioning, warranting further study.

Alternatively, the sex-specific differences are not due to maternal transfer, but due to sex-specific hormone metabolism by the embryo already in the first 3 days of incubation, depending on differential transfer of other yolk components that are influenced by male quality. Indeed, avian embryos heavily metabolize maternal androgens very early in development (Kumar et al., 2018a, 2018b; Paitz et al., 2011; Paitz and Casto, 2012). Again, this opens a new avenue for further research.

In conclusion, our results suggest that sex-specific maternal hormone transfer to eggs is influenced by male traits. These results open new avenues for further studies on both how and why mothers differentially transfer androgens in eggs in relation to offspring sex and mate quality and how ejaculate T affects male reproductive success and female reproductive decisions.

Ethical note

The procedures followed the relevant guidelines and regulations of the animal welfare committee of the University of Groningen and were approved by the committee under DEC license 6710B-001, 2015. All handling and treatment of animals were carried out by experienced scientist with a license (Certificate number 685412, DG VGZ/VVP (Strct.135), 25 January 2013), and animal caretakers, to perform animal experiments. The welfare of all birds was assessed on a daily basis. No injuries were observed after the short staged dyadic agonistic encounters (fights).

Author contributions

A.L., B.R., and T.G.G. designed the experiment. A.L. performed the experiment. A.L., B.R., and T.G.G. analysed the data. A.L. wrote the first draft of the manuscripts. B.R. and T.G.G. wrote with A.L. the final version. All authors read and approved the final manuscript.

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Data accessibility

Our data will be deposited at Dryad.

Declaration of competing interest

The authors declare to have no potential conflicts of interests (financial or nonfinancial) that are directly or indirectly related to the research.

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