

## University of Groningen

### Don't underestimate father

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DOI:  
[10.33612/diss.97045753](https://doi.org/10.33612/diss.97045753)

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*Document Version*  
Publisher's PDF, also known as Version of record

*Publication date:*  
2019

[Link to publication in University of Groningen/UMCG research database](#)

*Citation for published version (APA):*

Lelono, A. (2019). *Don't underestimate father: Effects of cryptic and non-cryptic paternal traits on maternal effect in a species without paternal care*. [Thesis fully internal (DIV), University of Groningen]. Rijksuniversiteit Groningen. <https://doi.org/10.33612/diss.97045753>

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# Chapter 2

Female reproductive  
investment in relation to male  
attractiveness in red junglefowl  
(*Gallus gallus gallus*)

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*Submitted*

## Abstract

Mothers are predicted to invest differentially in offspring from partners of different quality in order to optimize their fitness. A recent meta-analysis showed that females in female-care only species do so by investing more in egg mass when mated with attractive males rather than in other reproductive traits. We found some discrepancies with this result after reviewing all the literature available. Here, we report on these discrepancies and on an experiment we performed on the relationship between male comb size, a signal for attractiveness, and maternal reproductive investment in clutch production and offspring development in red junglefowl. We randomly mated females with a male with a large- or a small-comb and let them produce a clutch and raise the brood. After this first clutch, we paired up females with a male of opposite comb size and allow them produce a second brood. We found that females paired with large-comb males produced eggs sooner after pairing than females mated with small-comb males, but we did not find differences in egg mass or clutch size. Male comb size also affected growth and body condition, but only in daughters. Twenty four week old daughters of large-comb males were heavier and in better condition. These results support the positive differential allocation hypothesis (DA) for mate quality dependent reproductive investment rather than negative DA in which females would invest more in offspring sired from poor-quality males. This outcome adds to the discrepancies we found in the literature that warrants more study.

## Introduction

If there is variability in mate quality, life history theory predicts that reproductive strategies will evolve that allow individuals to adjust their reproductive investment according to the expected fitness returns (Burley, 1986; Sheldon, 2000). Although in many species males invest heavily in reproduction through the acquisition and maintenance of territories and mates, females, in general, are limited by the number of eggs they can produce. Females invest initially more in the actual production of zygotes, embryos, and new-borns both through a more substantial investment in the ovum while males do in spermatozoids, as well as during incubation or gestation. It is therefore expected that the physiological and behavioural reproductive strategies to adjust reproductive investment to partner quality are especially pronounced in females, certainly in species where the male does not contribute much to embryonic development or postnatal care.

Reproductive strategies involve decision making about 'when to reproduce', 'how many offspring to produce', and 'how much to invest in the offspring' in order to optimize current and future reproductive success. If females of equal reproductive potential end up with mates of different quality, a dichotomy in reproductive strategies will emerge: 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 for having a low-quality father (the compensation hypothesis), (Gowaty, 2008; Gowaty et al., 2007). The strategy chosen may depend on several aspects such as the species specific division of labour between the sexes in raising offspring (Horváthová et al., 2012). Currently, these two hypotheses are seen as one continuum and are now mostly referred to as positive and negative DA, where positive DA seems to be most common in birds (Haaland et al., 2017; Harris and Uller, 2009; Kindsvater and Alonzo, 2014).

The females' non-genetic contributions that influence offspring development, collectively known as maternal effects (Mousseau and Fox, 1998), have been shown to be related to paternal attractiveness in several avian species but not always in a consistent manner (for a review see (Horváthová et al., 2012; Von Engelhardt and Groothuis, 2011). This maternal effect study is of special interest as it consists of a quantitative meta-analysis, which showed that, in favour of the positive DA over

negative DA, avian females invest more in current reproduction when paired with attractive high-quality males. From this analysis, it also emerged, but with small to moderate effect sizes, that females in species with bi-parental care invest in producing more eggs, whereas females of species with female only care invest in larger eggs when paired with high-quality attractive males. There was no effect on 'when to produce' except an advance in clutch initiation of first year female mallards (*Anas platyrhynchos*) when mated with more attractive drakes (Sheppard et al., 2013). The investment in more cryptic components such as the embryonic exposure to immune stimulants and exposure to androgens, has no consistent effect. This latter finding may in itself not be evidence against the positive DA hypothesis since it is unknown whether adding hormones or immuno-enhancing molecules are costly to females both in the present or in future reproductive events (Groothuis and Schwabl, 2008; Sheldon, 2000). However, the results of this meta-analysis with respect to bi-parental versus uniparental care should be taken with some cautions as the meta-analysis contained only 8 studies on 5 species that show female only care: the chinese quail *Coturnix chinensis*, (Uller et al., 2005), the grey partridge *Perdix perdix*, (Garcia-Fernandez et al., 2010), the houbara bustard *Chlamydotis undulata*, (Loyau and Lacroix, 2010), the mallard *Anas platyrhynchos*, (Bluhm and Gowaty, 2004; Cunningham and Russell, 2000; Giraudeau et al., 2011), and the peafowl *Pavo cristatus*, (Loyau et al., 2007).

We found 7 additional studies, 4 older than at the time of publication of this meta-analysis (Petri and Williams 1993 (peafowl), Forkman and Corr 1996 (domestic chicken – *Gallus gallus domesticus*), Rintamaki et al. 2000 (black grouse - *Tetrao tetrix*), Parker 2003 (red junglefowl – *Gallus gallus gallus*) and 3 of similar date or newer (Cucco et al. 2011, Alonso-Alvarez et al. 2012, both in the red legged partridge – *Alectoris rufa*, Sheppard et al. 2013 and mallard), that relate female reproductive investment to male quality in female care only species. In contrast to the results of the meta-analyses (Horváthová et al., 2012), four of these studies report on egg size (mass/volume) and all report that there is no relationship between attractiveness and egg size (Petri & Williams 1993, Parker 2003, Cucco et al. 2011, and Alonso-Alvarez et al. 2012) with the exception of Forkman and Corr (1996) that did find a positive relationship (but see below). Also, four studies report on the relationship with laying date, 2 of these (Petri and Williams 1993, Cucco et al. 2011) report no relationship and two (Alonso-Alvarez et al. 2012, and Sheppard et al. 2013) indicate an advanced laying date when mated with more attractive males. One of the studies

(Forkman and Corr, 1996) showed, contrary to expectation, that although female leghorns (*Gallus gallus domesticus*) invested more in egg mass when mated with males with more symmetrical wattles, a trait that may be related to attractiveness, there was a negative relationship between wattle size and the number of fertilized eggs produced. Moreover, in four of these studies, the number of eggs laid increased with male attractiveness (Petri and Williams 1993, Rintamaki et al. 2000, Parker 2003, and Alonso-Alvarez et al. 2012). These extra results make the generalization of the conclusion that females mate with attractive males invests more in egg mass rather than the number of eggs in female only care species, less convincing.

Given the inconsistent results presented above, the current study aimed was to add new data to answer the question whether in species where only females perform offspring care there is the differential investment in current reproduction that is related to mate quality. Many traits may signal quality of males; these may be behavioural traits, such as food provision and vigilance (Pizzari 2003, Wilson et al. 2008) or morphological traits such as the size of the comb. Comb size in junglefowl positively affects female mate choice (Collias and Collias, 1996; Johnsen and Zuk, 1995; Parker and Ligon, 2003; Zuk et al., 1995). Moreover, comb size is heritable (Parker, 2003) and is related to social dominance (Parker et al., 2002; Parker and Ligon, 2003). Furthermore, increased comb size is related to decreased immunocompetence (Zuk et al. 1995), which indicates that only high-quality males can bear the cost of a decreased immunocompetence (Zuk 1992). These relationships indicate a correlation between phenotypic quality and attractiveness where males with large-comb are of higher quality because they are more dominant and have fewer circulating lymphocytes (Folstad and Karter, 1992; Verhulst et al., 1999; Zuk et al., 1995) and are therefore more attractive to females than small-comb males. We therefore firstly paired up hens with roosters with a large-comb or a small-comb (selected at random) and allowed them to produce a clutch and raise a brood to see whether rooster quality influenced female reproductive investment in the clutch. Importantly, we also analysed whether offspring development was positively affected when females were mated with high-quality males, as was the case in the mallard (Bluhm and Gowaty, 2004), the houbara bustard (Loyau and Lacroix, 2010), and the peafowl (Loyau et al., 2007; Petrie and Williams, 1993). Studies often do not report on offspring development even though this is an important outcome of reproductive investments. Subsequently, we switched mating partners: Hens those were first paired up with a large-comb (high-quality) rooster were now paired up

with a small-comb (low-quality) rooster and vice versa. Hens were then allowed to produce a second clutch and brood. Here we report on the relationship between male quality (as reflected by comb size) and 'when to reproduce' (clutch initiation time), 'how many offspring to produce' (clutch size), 'how much to invest in offspring' (egg mass), and offspring growth into early adulthood.

## Methods

### Experimental animals

In this experiment, we used 14 pairs of sexually naive captive bred red junglefowl (*Gallus gallus gallus*) from our aviary at the University of Groningen The Netherlands. All hens and 10 roosters were two to three years old; the other 4 roosters were 1 year old. The experiment was initiated in late spring of 2013 and lasted until autumn of 2013. Prior to the experiment, hens and roosters were housed in two single sex groups in separate outdoor aviaries.

### Experimental design

Two types of biometrical measurements of all parental birds were taken at the beginning of pairing the birds with each other in order to assess male quality and balance hens over the two experimental groups. We measured body mass to the nearest gram and comb surface area (comb size) was determined as follows: Firstly, we attached a removable circular sticker (diameter 0.8 cm) to the comb of the males and then photographed all male comb using a digital camera (Canon SX 500 IS: diaphragm 4,3 – 129,0 mm). Pictures were made only of the left side of the rooster heads. The images were imported in GIMP 4.8, and the comb were manually outlined, and the number of pixels in the area was determined. Comb size was then determined by contrasting this number of pixels against the number of pixels of the sticker (from the same photograph) with a known surface area determined. Similarly, comb length was determined by measuring the longest straight line from the upper beak (front comb) to the end of the comb. Furthermore, we determined comb colour using a spectrophotometer and procedure identical to that was described in Riedstra et al. (2013). Roosters were then ranked by of comb size and the 7 birds with the largest comb were qualified as 'large-comb' roosters, the remaining 7 with

the smallest comb size as 'small-comb' roosters. Male phenotypic characteristics are described in Table 1.

We then paired up females with either a large-comb male or a small-comb male in such a way that the average body mass of the two female groups did not differ (see Table 2). These pairs were each housed in one of 14 identical aviaries (1.5 × 3 × 2.5 m (l × w × h)) with ad-libitum access to water and food (standard chicken pellets), a dustbathing area, a perch, and a nest site. All pairs were checked daily for egg production. When eggs were produced, they were weighed and marked with a non-poisonous felt-tipped pen for identification purposes and placed back in the nest and left for hatching.

### Chick housing and growth

On the day of hatching, each chick was weighed, and individually colour marked using flexible rubber leg bands. After that, they remained in the home cage condition with both parents present for five weeks. We choose for this setup because the males' phenotypic characteristics could reinforce the hens' reproductive decisions during the total period of incubation and chick rearing, even though females (both red junglefowl and feral chickens) living in natural conditions are generally classified as solitary when having small dependent chicks (Collias & Collias 1996, McBride et al. 1969). They may however frequently encounter males or other broody females during foraging because their home ranges and territories overlap and it has even been observed that males tidbit to chicks (McBride et al. 1969), which we also found on only a few occasions. Casual observations indicated that the males in our experiment remained at a distance from the broody hen and her chicks and hardly interacted with them.

After three weeks the leg bands were replaced by a numbered metal wing tag for permanent identification. At the age of five weeks, all chicks of the first reproductive attempt were relocated to a single large aviary. The chicks from the second clutch were also relocated at the age of 5 weeks, to a different but identical aviary. At the age of three months, the roosters and hens of the first clutch were separated and divided into two aviaries. Hens of the second clutch were housed with the hens of the first clutch when they were three months old, whereas the roosters of the second clutch were housed in a separate aviary to avoid escalation of aggression. At the age of 24 weeks (about 5 and a half months), we determined body mass and tarsus length (using sliding callipers) of all offspring.



## Reversal of treatment

After the first brood was raised and removed, the treatment was reversed after at least one week of recuperation: females those were first paired up with a large-comb male were now paired up with a small-comb male and vice versa. At this point, biometrical measurements of the males were taken again, and the next procedures were done as mentions above (experimental design). Since females first mated with large-comb males, initiated clutch laying a week earlier than females paired with small-comb males (see results section), they had on average a week longer to recuperate from the first reproductive attempt.

## Statistical analysis

Based on the distribution of the raw data and the residuals of models we used parametric tests.

We first analysed whether our divisions between large- and small-comb males resulted in two groups that differed in comb size (surface area), and length whether other phenotypic characteristics (comb colour and body mass) differed between the two groups using independent t-tests (see Table 1). Similarly, we then analysed whether female body mass differed between the two treatments at both the onset of the first and the second reproductive attempt (see Table 1).

To analyse female reproductive performance (clutch size, average egg mass and clutch size) we conducted a multivariate test. The test analysed on the difference between clutch initiation time, average egg mass and clutch size between the reproductive attempt with a large-comb male and the attempt with a small-comb male, using a general linear model. Furthermore, we analysed the difference in hatching success separately (because our main predictions were on clutch initiation time, clutch size, and average egg mass) using a one sample t-test. Two females, those firstly paired with a small-comb and secondly with a large-comb male, did not reproduce at all within the given time frame of the experiment and were removed from all analyses on reproductive investment because no other data then clutch size (0) was available. Furthermore, two hens produced a clutch in both conditions, but none of their eggs showed embryonic development and did not hatch. These clutches were omitted from the analyses on hatching success.

The data of chick body mass at hatching and 24 weeks old was analysed using generalized linear mixed models on body mass averaged per mother over same-sex siblings, mother as a random factor and male quality, offspring sex and the

interaction between male quality and offspring sex as fixed variables. Here we also performed 2 post-hoc tests within daughters and 2 within sons because there was a significant interaction effect on growth (body mass and condition), of offspring sex and male quality. All statistical analyses were performed in SPSS 24.

## Results

### Phenotypic differences between males and between females

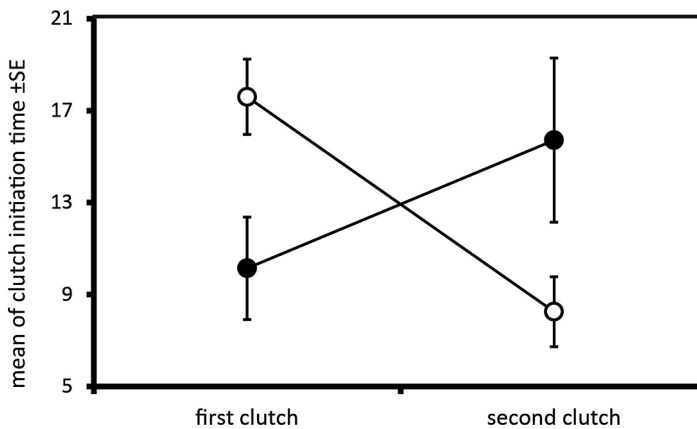
Our selection of comb size resulted in significant differences in comb size and comb length between the two groups of males (see Table 1). Large-comb males had comb surface areas about twice the size of the small-comb males, and comb length was about 1.3× longer. Furthermore, large-comb males were heavier than small-comb males in the first reproductive attempt, but this was not so in the second. Overall there were no differences in comb colour characteristics between the groups (see Table 1). There was also no effect of treatment, reproductive attempt (first or second clutch) on female body mass (Table 1, first row). Therefore, our objective was reached namely that the treatment groups only differed in male comb size.

Table 1 | Phenotypic characteristics (mean  $\pm$ SE in brackets) of large – (N = 7) and small-comb roosters (N = 7): Body mass is expressed in gram, comb size in cm<sup>2</sup>, comb length in mm, brightness as the percentage reflectance, chroma is dimensionless, and hue is expressed in nm

male comb size	first clutch				second clutch			
	large	small	T	p	large	small	T	p
male body mass	1232.3 (68.5)	1043.3 (42.0)	5.06	0.046	1131.0 (61.6)	1005.2 (27.3)	2.78	0.121
comb size	18.9 (0.98)	10.5 (1.09)	32.0	< 0.001	19.0 (0.89)	10.0 (1.20)	37.4	< 0.001
comb length	78.3 (2.38)	59.8 (2.11)	32.7	< 0.001	78.0 (2.13)	60.2 (2.31)	32.8	< 0.001
brightness	9.4 (0.71)	9.3 (0.93)	0.02	0.885	9.3 (0.52)	11.2 (1.61)	1.45	0.252
chroma	1.29 (0.14)	1.41 (0.16)	0.32	0.580	1.32 (0.08)	1.36 (0.21)	0.03	0.859
hue	624.6 (3.41)	611.9 (8.83)	2.01	0.183	612.6 (5.93)	597.7 (14.02)	1.11	0.311
female body mass	874.1(29.2)	828.8(36.9)	0.96	0.350	833.4 (19.5)	859.3 (20.0)	0.85	0.370

## Female reproductive investment

There was no effect of male comb size on either clutch size (multivariate GLM:  $F_{(1,11)} = 0.02$ ,  $p = 0.905$ ) or egg mass (multivariate GLM:  $F_{(1,11)} = 0.152$ ,  $p = 0.705$ ). However, hens started laying egg approximately one week sooner when paired up with a large-comb rooster than a small-comb rooster (Figure 1 multivariate GLM:  $F_{(1,11)} = 5.05$ ,  $p = 0.048$ ). There were no differences in hatching success (one sample t-test  $T = 0.583$ ,  $P = 0.576$ ), see Table 2 for reproductive variables.



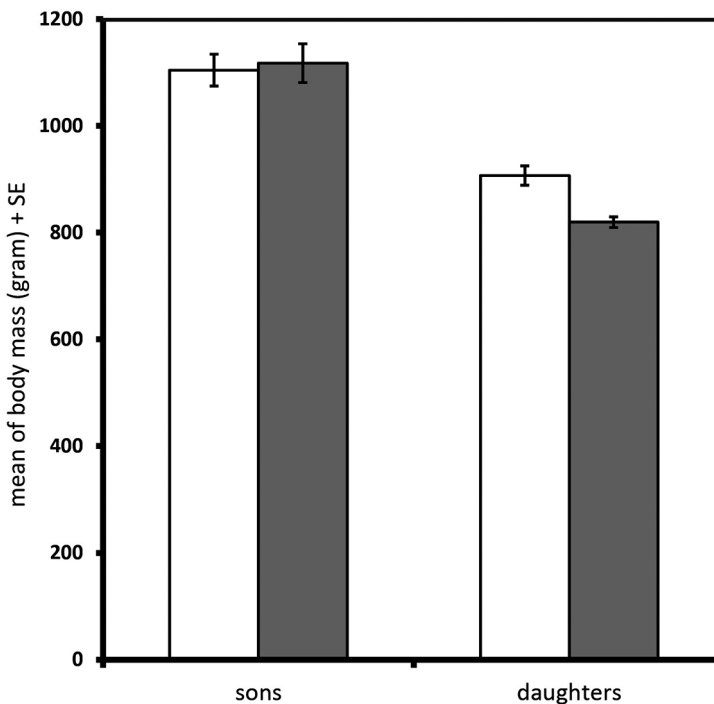
**Figure 1** | Mean of clutch initiation time (in days  $\pm$ SE) of hens that were first paired up with a large-comb male and then with a small-comb male are represented by the closed dots. The reverse treatment is represented by the open dots

**Table 2** | Reproductive performance (mean  $\pm$ SE., and sample size in brackets) of females paired up firstly with large- and secondly small-comb roosters or vice versa

male comb size	first clutch		second clutch	
	large	small	small	large
clutch initiation time (days)	10.1 $\pm$ 2.2 (7)	17.6 $\pm$ 1.6 (5)	15.7 $\pm$ 3.5 (7)	8.2 $\pm$ 2.01 (4)
clutch size	6.0 $\pm$ 0.6 (7)	6.6 $\pm$ 1.2 (5)	5.7 $\pm$ 0.7 (7)	6.0 $\pm$ 0.4 (4)
average egg mass	30.8 $\pm$ 1.2 (7)	31.8 $\pm$ 0.8 (5)	32.4 $\pm$ 1.2 (7)	33.5 $\pm$ 0.6 (4)
hatching success (percentage)	63.6 $\pm$ 10.9 (5)	71.1 $\pm$ 8.5 (5)	64.7 $\pm$ 10.3 (5)	50.0 $\pm$ 9.5 (4)
hatchling mass	23.3 $\pm$ 1.2 (22)	24.0 $\pm$ 0.8 (22)	21.0 $\pm$ 1.1 (16)	22.8 $\pm$ 0.5 (12)

## Offspring growth

There was no effect of male comb size, offspring sex or the interaction between male comb size and offspring sex on body mass at hatching (GLMM:  $F$ -values  $< 1.44$ ,  $p$ -values  $> 0.706$ ). Twenty-four weeks after hatching, sons had outgrown daughters (GLM,  $F_{(1,16)} = 37.3$ ,  $p < 0.001$ ), and there was no direct effect of male comb size ( $F_{(1,15.1)} = 0.29$ ,  $p = 0.599$ ), but there was an interaction between male comb size and offspring sex on body mass ( $F_{(1,15.1)} = 5.29$ ,  $p = 0.036$ ). Sons of large-comb males ( $N = 13$ ) did not differ from sons from small-comb males ( $N = 19$ ; figure 2; GLMM;  $F_{(1,2.4)} = 6.66$ ,  $P = 0.101$ ), but daughters ( $N = 13$ ) from large-comb males were heavier than daughters ( $N = 14$ ) of small-comb males ( $F_{(1,11)} = 11.82$ ,  $p = 0.006$ , Figure 2). Furthermore, there was an interaction between male comb size and offspring sex on body condition (body mass / tarsus length;  $N = 59$ ,  $F_{(1,15.4)} = 7.98$ ,  $p < 0.013$ ): daughters of small-comb fathers had a lower condition score than daughters of large-comb fathers ( $12.3 \pm 0.19$  vs.  $14.1 \pm 0.47$ ;  $N = 27$ ,  $F_{(1,11)} = 13.82$ ,  $p = 0.003$ ), whereas there was no difference in sons ( $13.9 \pm 0.78$  vs  $13.3 \pm 0.47$ ;  $N = 32$ ,  $F_{(1,3.6)} = 0.881$ ,  $p = 0.406$ ).



**Figure 2** | Mean of chicks body mass (in gram  $\pm$ SE.) of sons and daughters sired by large-comb roosters (dark bars) or small-comb roosters (white bars) 24 weeks after hatching

## Offspring growth

There was no effect of male comb size, offspring sex or the interaction between male comb size and offspring sex on body mass at hatching (GLMM:  $F$ -values  $< 1.44$ ,  $p$ -values  $> 0.706$ ). Twenty-four weeks after hatching, sons had outgrown daughters (GLM,  $F_{(1,16)} = 37.3$ ,  $p < 0.001$ ), and there was no direct effect of male comb size ( $F_{(1,15.1)} = 0.29$ ,  $p = 0.599$ ), but there was an interaction between male comb size and offspring sex on body mass ( $F_{(1,15.1)} = 5.29$ ,  $p = 0.036$ ). Sons of large-comb males ( $N = 13$ ) did not differ from sons from small-comb males ( $N = 19$ ; figure 2; GLMM;  $F_{(1,2.4)} = 6.66$ ,  $P = 0.101$ ), but daughters ( $N = 13$ ) from large-comb males were heavier than daughters ( $N = 14$ ) of small-comb males ( $F_{(1,11)} = 11.82$ ,  $p = 0.006$ , Figure 2). Furthermore, there was an interaction between male comb size and offspring sex on body condition (body mass / tarsus length;  $N = 59$ ,  $F_{(1,15.4)} = 7.98$ ,  $p < 0.013$ ): daughters of small-comb fathers had a lower condition score than daughters of large-comb fathers ( $12.3 \pm 0.19$  vs.  $14.1 \pm 0.47$ ;  $N = 27$ ,  $F_{(1,11)} = 13.82$ ,  $p = 0.003$ ), whereas there was no difference in sons ( $13.9 \pm 0.78$  vs  $13.3 \pm 0.47$ ;  $N = 32$ ,  $F_{(1,3.6)} = 0.881$ ,  $p = 0.406$ ).

## Discussion

In this study, we assessed whether the comb size of red junglefowl roosters affected female reproductive investment and offspring development. Comb size is a proxy for male quality, since this feature is a proven indicator for male attractiveness, is related to social dominance, reduces immunocompetence (which only good-quality individuals may be able to bear the costs off) and is heritable (Collias and Collias, 1996; Johnsen and Zuk, 1995; Johnsen and Zuk, 1996; Parker, 2003; Parker and Ligon, 2003). Roosters in our population with large-comb (both in maximum length and size) were heavier but did not differ in comb colour from small-comb (low-quality) roosters. Based on the assumption that in general, positive DA is most common in birds (Harris and Uller, 2009; Ratikainen and Kokko, 2010) and that females of female care only species invest in egg size or mass when paired with attractive males (Horváthová et al., 2012), one may expect that females paired with large-comb males to produce heavier eggs. However, as suggested by additional literature (see introduction), we did not find this effect on egg mass. Neither did we find an effect on clutch size. However, we did find that hens paired with large-comb

males, initiated egg laying sooner. In general, early breeding is best in seasonal environments (Rowe 1994, Verhulst et al. 1995).

Our results are therefore in line with a) the parental quality hypothesis where the earliest breeders (Rowe, 1994) may be those of highest quality (Low et al., 2015; Verhulst and Nilsson, 2008; Wardrop and Ydenberg, 2003), and b) with the positive DA hypothesis where the attractiveness of the partner stimulates reproductive investment (Cunningham and Russell, 2000; Harris and Uller, 2009). Disregarding possible differences in behaviour between the males with different comb sizes (Pizzari 2003, Wilson et al. 2008), there were no apparent phenotypic differences between the females in the two treatment groups, and they did not invest differentially in egg mass or clutch size. Both Wilson et al. (2008) and Pizzari (2003) did not find strong positive correlations between behaviours that predict male reproductive success and comb size, and we may, therefore, assume that these behaviours were randomly divided over our treatments, possibly having confounding effects on our results, but nevertheless the perceived male quality (comb size) must, therefore, have been the main factor affecting clutch initiation time.

Furthermore, hens mated with large-comb males produced heavier daughters with a higher condition score, but no such effects were found in sons. The absence of effects in sons was unexpected since Parker (2003) already found a positive effect of paternal comb size on offspring condition. Parker (2003) also showed that paternal genetics may underlie differences in development but could not rule out non-genetic paternal effects. Parker (2003) suggested that such non-genetic paternal effects could be transmitted via the chemical compounds in the roosters' ejaculates. A novel idea that has not received much attention.

The ejaculate of cocks is known to contain several steroid hormones (Anderson and Navara 2011). Hens have steroid receptors in the oviduct (Takeda et al. 1990, Yoshimura et al. 2000, Walters et al. 2010, Chang et al. 2013) and also deposit androgens in their eggs (Riedstra et al. 2013). We recently showed that testosterone concentrations in the ejaculate differ between large- and small-comb males (Lelono et al. 2019). This opens the possibility that the earlier initiation of egg laying may have been caused by differences in ejaculate composition between large- and small-comb males, which open for further testing. It is also conceivable that the testosterone in the ejaculate enters the egg during fertilization, affecting chick growth, although this then should have a sex-specific effect which is not unusual for these maternal hormones (Groothuis et al., 2019).

Contrary to Parker (2003) our set up was not designed to separate maternal responses from paternal effects. Therefore, maternal effects such as sex-specific yolk testosterone deposition may explain in our results, since a) this steroid is known to enhance early growth (Grootuis et al. 2005b), b) differential hormone deposition in relation to paternal quality is present in avian species (Gil et al. 1999, 2004, Rutstein et al. 2005, von Engelhardt et al. 2006, Williamson et al. 2006, Garcia-Fernandez et al. 2010, Loyau and Lacroix 2010), although some other studies did not find this (reviewed in Kingma et al. 2009, Cucco et al. 2011, Horvathova et al. 2012), and c) there is evidence that avian mothers can deposit testosterone in the yolk depending on the sex of the embryo (Muller et al. 2002, Badyaev et al. 2005, Rutstein et al. 2005, Gilbert et al. 2005, Pariser et al. 2012). From this, the intriguing hypothesis emerges that maternal (sex-specific) hormone deposition may be mediated by a cryptic paternal trait, namely the ejaculate hormone composition of fathers (Lelono et al., 2019a). Heavier daughters sired by large-comb males support the positive DA hypothesis. Nevertheless, based on the current results we did not find evidence in the development of sons that supports either the positive or negative DA hypothesis. In conclusion: In line with previous studies, we found that paternal attractiveness affects female reproductive investment and offspring growth in red junglefowl. Whether this effect is caused purely by visual perception of the male comb by females or perhaps via mechanisms involving the chemical substances in the ejaculates of males, as suggested as a possible path way by Parker (2003), is currently under investigation. However, although our results support the positive DA hypothesis, because females mated with large-comb males produce eggs earlier than females mated with small-comb males, we found no evidence that in the red junglefowl, females invest more in egg mass as was found by Horváthová et al. (2012). These results together with the studies presented in the introduction, therefore, weaken the generalized conclusion that females in female care only species invest more in egg mass when mated with attractive/good-quality males.

## **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 6710A, 2013.

All handling and treatment of animals were carried out by experienced scientist with a licence (Certificate number 685412, DG VGZ/VVP (Stcrt.135), 25 January 2013), and animal caretakers, to perform animal experiments. The welfare of all birds was assessed on a daily basis.

## **Funding**

This work is supported through a Direktorat Pendidikan Tinggi (DIKTI) Scholarship, The Ministry of Research, Technology and Higher Education, The Republic of Indonesia and the University of Groningen The Netherlands to A.L. and T.G.G.

## **Author contributions**

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

## **Acknowledgments**

Special thanks to Saskia Helder for her assistance in animal caretaking.





**“Semar”** Abdillah Ronggo Asmoro 2015