

Ejaculate Expenditure Strategies in a Polygynandrous Bird, the Red Junglefowl, *Gallus gallus*



Yunke Wang
The St Catherine's College
University of Oxford

Supervisor: Professor Tommaso Pizzari
Department of Zoology

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THESIS ABSTRACT

Plastic strategies of ejaculate expenditure are a fundamental aspect of male reproductive fitness, especially in polygynandrous species where multiple mating opportunities expose males to the risk of sperm depletion, and sperm competition favours large, high-quality ejaculates. Little however remains known about factors that modulate ejaculate expenditure behaviour. In this thesis, I explored how four different factors affect male ejaculate expenditure plasticity in the polygynandrous red junglefowl, *Gallus gallus*: temporal effects, male social status, female reproductive quality and female sexual novelty.

Firstly, I show that male ejaculate expenditure has a marked seasonal pattern, gradually peaking in June and decreasing later in the season. However, contrary to previous studies in domestic fowl, ejaculate expenditure patterns were largely insensitive to the time of the day (morning, midday, afternoon). Secondly, social status mediates access to females and the level of sperm competition faced by the ejaculates of a male. Dominant males have a higher copulation success and face a lower risk of sperm competition than subordinate males. Inconsistent with the findings of previous work in domestic fowl, I found no evidence that male social status predicted male's ejaculate expenditure nor did I find a significant change in ejaculate expenditure resulting from a change in a male's social status. Thirdly, I found that males exposed to large-combed females have higher ejaculate investment than males exposed to small-combed females, which suggests that males have the ability to assess female reproductive quality, consistent with previous work in fowl. Importantly, a male's ejaculate expenditure in the intermediate-combed female in the final trial was similar to his expenditure in the previous four trials such that the intermediate-combed female received more sperm from males that had previously mated with large- versus small-combed females. These results suggest that males may have a carry-over effect where previous experience influences ejaculate expenditure in the current mating opportunity. Fourthly, we

experimentally confirmed previous findings that males have a preferential sperm investment in sexually novel females (the Coolidge effect). Males were also more likely to mate with a novel female and ejaculated sooner than males exposed to an experimental control. In addition, we found that in the first trial, when males were naïve with respect to experimental treatments, expenditure patterns were similar across treatments and showed no evidence for a preferential investment in novel females which suggests that males may require previous experience to generate an expectation of the novelty of the female in the next mating opportunity and plan ejaculate expenditure in advance (i.e. reserve some sperm for the second female). There was also some evidence that males exposed to different treatments had different patterns of ejaculate expenditure in the final trial where all males were exposed to two females sequentially. However, the extent to which these differences were explained by previous experience is less clear. Collectively, the findings of these studies demonstrate that the temporal change, social status, female quality and female novelty may have profound effects on plastic male ejaculate expenditure.

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AUTHORS CONTRIBUTIONS

Chapter 2: Seasonal and circadian patterns of male ejaculate expenditure

Yunke Wang, Ellen O'Brien, Ella Glover, Evan Deakin, Romulo Carleial, Tommaso Pizzari

YW and TP conceived and designed the study. YW collected samples for analysis with the help of EBO, EG, ED. YW and RC analysed and interpreted the data. YW wrote the chapter.

Chapter 3 Patterns of ejaculate expenditure in relation to male social status

Yunke Wang, Ellen O'Brien, Tommaso Pizzari

YW and TP conceived and designed the study. YW collected samples for analysis with the help of EBO. YW analysed and interpreted the data. YW wrote the chapter.

Chapter 4 Cryptic male choice for ornamented females

Yunke Wang, Ella Glover, Tommaso Pizzari

YW and TP conceived and designed the study. YW collected samples for analysis with the help of EG. YW analysed and interpreted the data. YW wrote the chapter.

Chapter 5 The influence of past experience on male investment in a novel female

Yunke Wang, Anamaria Sluganovic, Ellen O'Brien, Liisa Veerus, Tommaso Pizzari

YW and TP conceived and designed the study. YW collected samples for analysis with the help of AS, EBO, LV. YW analysed and interpreted the data. YW wrote the chapter.

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Chapter 1: Introduction

Sexual selection

Intrigued by male elaborate traits, Darwin first proposed the concept of sexual selection to explain the widespread sexual dimorphism in the animal kingdom ¹. Darwin defined sexual selection as the process that favours individuals carrying traits that confer an advantage in intrasexual competition over access to mates and reproductive opportunities. Darwin proposed that sexual selection operates through both inter- and intrasexual episodes. Intersexual episodes occur through the process whereby individuals of the one sex select members of the opposite sex as reproductive mates, through mate choice. Intrasexual episodes arise through the direct competition of individuals of the same sex for mating opportunities. Sexual selection is usually more pronounced in males, due to anisogamy, and resulting differences in parental investment and biased operational sex ratio ². Thus, female choice and male competition are studied most intensively. It is well established that female choice and male competition for access to female partners promotes the evolution of a wide array of male traits ^{1,3}. Postmating traits are selected for the ability of males and females to manipulate the paternity of offspring.

Polyandry

Polyandry refers to any mating system where individual females mate with multiple males, which causes sexual selection to continue after mating. In 1970, Parker argued that when polyandry occurs, the sperm of different males will compete for fertilisation of the same set of ova (sperm competition) ⁴. Females were not expected to benefit from mating multiply for a long time since Bateman argued that males reproductive success is largely dependent on the number of females inseminated (i.e. mating success), while female reproductive success typically does not increase with the number of mates once fertilisation of a set of eggs is guaranteed by few copulations ⁵. Thus, males, but not females, were expected to be under stronger sexual selection to mate with multiple

mates. However, throughout the animal kingdom, females of many species typically copulate with multiple males within a single reproductive cycle ⁶. This may be explained by the possibility that, in contrast to Bateman's expectations, recent studies show that females too may obtain some fitness benefits from polyandry. The potential benefits that females may gain from polyandry are divided into direct benefits and indirect benefits.

Females may benefit from polyandry directly, through the male provision of nuptial gifts, protection, fertilisation insurance etc. In the spiders, *Pisaura mirabilis*, males bring nuptial gifts containing fresh prey. The nuptial gift is nutritious and enhances female's fecundity ^{7,8}. There are also benefits derived from male mate guarding against sexual harassment, protection, territory sharing and paternal care ⁹⁻¹¹. Furthermore, polyandry may ensure fertility when males fail to provide sufficient competent sperm ¹².

In addition to direct benefits, a number of genetic benefits have been proposed for the evolution of polyandry. Firstly, polyandry can have a positive effect on embryo viability (reviewed by ¹³) by increasing the genetic diversity of sperm the females receive, which in turn reduces potential risks of fertilisation by genetically incompatible sperm ¹⁴. The major histocompatibility complex (MHC) is a class of genes that provides resistance to specific infectious diseases in vertebrates. MHC-heterozygous offspring may have enhanced immunocompetence ¹⁵. There is some evidence suggesting that in some species antigens are expressed on the surface of spermatozoa which enables females to recognise the MHC haplotype of individual sperm^{10,16}. Eggs of the three-spined stickleback, *Gasterosteus aculeatus*, bias toward sperm with MHC immunogens, resulting in an intermediate level of individual MHC diversity which confers highest pathogen resistance for the offspring¹⁷.

Secondly, high-quality ejaculate is more likely to achieve fertilisation success ¹².

Because ejaculate traits are heritable ¹⁸ thus sons of polyandrous females are more likely to inherit high-quality ejaculate traits from their fathers and achieve higher fertilisation success. For example, semen traits from in German warmblood stallions, *Equus ferus*, show moderate heritability ¹⁹.

Limited ejaculate

All else being equal, two key components tend to determine the outcome of the sperm competition across different taxa: the relative number of sperm inseminated by rival males in the same female and the relative fertilising efficiency of competing ejaculates ²⁰⁻²⁵, determined by ejaculate traits such as sperm motility ²¹. Ejaculates are costly to produce ²⁶⁻²⁸, and in numerous species the sperm reserves of males can become depleted by mating, which limits their reproductive success. For example, in the golden orb-web spider, *Nephila clavipes*, sperm is permanently depleted after a single mating²⁹.

The fertilising advantage resulted from inseminating more and/or higher quality sperm, is expected to lead to the evolution of male strategies of differential ejaculate investment. Because females mate polyandrously, males face sperm competition, which promotes male ejaculate expenditure. Males are therefore expected to evolve strategies of economical ejaculate expenditure to maximise their reproductive success within the constraints imposed by sperm production ^{25,30,31}. Specifically, males are expected to invest ejaculate strategically according to the reproductive payoffs associated with different mating opportunities. Behavioural plasticity, the ability to act differently depending on the context of a mating opportunity, enables individuals to respond rapidly to changes in the environment and maximise fitness in an uncertain world ³². Therefore, males are driven to evolve fully reversible ejaculate allocation strategies to rapidly adjust to changes in the socio-sexual environment. Kelly and Jennions ³¹ found that males transfer larger ejaculates to higher-quality females. Sperm investment also increased when the number of rivals increased from 0 to 1. But as the number of rivals increased from 1, no evidence supports the general prediction that males often

decreased sperm allocation^{30,33}.

One prerequisite for strategic ejaculate expenditure is that males have the ability to partition sperm and seminal fluid proteins. There is increasing evidence that male mating behaviour exhibits great plasticity and can change rapidly to changes in the social environment. For example, in the fruit fly, *Drosophila pachea*, and Japanese pygmy squid, *Idiosepius paradoxus*, males can reduce the number of sperm in each ejaculate in order to be able to mate multiple times^{34 35}. Males not only have the ability to partition sperm across different mating events but they also have the ability to partition seminal fluid components.

There is increasing evidence that in addition to sperm effects, postmating sexual selection may be strongly influenced by seminal fluid products. Seminal fluid is the physiological non-sperm component of an ejaculate, which is mainly, but not exclusively, produced by sex accessory glands³⁶. Seminal fluid helps males to win the sperm competition by enhancing sperm swimming velocity and to manipulate female behaviours and physiological processes^{37 38,39 38 40 41 42 43}. A study in *D. melanogaster* has found that small males invest more seminal fluid proteins than large males, and males allocate more seminal fluid to larger and more fecund females⁴⁴. The study has shown that the common bedbugs, *Cimex lectularius*, males allocate 12% of their sperm number and 19% of their seminal fluid volume to each mating, suggesting that bedbugs would run out of seminal fluid before sperm, and that means male's mating rate is largely constrained by seminal fluid rather than sperm⁴⁵.

Male ejaculate expenditure strategy

A number of factors mediate male ejaculate expenditure strategies. Below, I consider: (i) seasonal/circadian patterns, (ii) male social status, (iii) female reproductive quality, and (iv) female novelty/availability.

(i) Seasonal/circadian patterns

Male's mating behaviour including ejaculate expenditure can vary at different time scales, for example, over the course of a year (seasonal) and over a day (circadian). Variance in these temporal patterns contributes to the evolution of ejaculate expenditure plasticity. Seasonal changes in sperm supplies can modulate ejaculate expenditure strategies. However, at different times in the breeding season, the mating prospects are different. Males also need to balance investment in current mating opportunities versus potential future mating opportunities. At the start of the breeding season, males have a good future mating prospect, they would be more prudent than at the end of the breeding season when the future mating opportunity is scarce. For example, males of the threespine stickleback, *Gasterosteus aculeatus*, a seasonal breeder, have flexible mating strategies at different times during the breeding season. In general, male stickleback are less prudent at the end of the breeding season ⁴⁶.

Mating behaviour can also be profoundly shaped by circadian patterns (reviewed by ⁴⁷). In temperate zone, the length of daylight is in continuous change and males can adjust their behaviours or physiological process according to daylight. For example, animals can synchronise their sperm migration within male reproductive tract according to daylight. In wild gypsy moth, *Lymantria dispar*, 50% of males had released sperm from the testes into the extragonadal sperm reserves (i.e. vasa deferentia) 3 hours before dusk⁴⁸. Extragonadal sperm reserves positively correlates with sperm investment in some species. For example, in Finewool Saxon Merino rams, *Ovis aries*, the number of sperm ejaculated daily is linearly correlated with extragonadal spermatozoan reserves ⁴⁹. Sperm depletion in the vas deferens could lead to decreasing sperm investment in Bengalese finch, *Lonchura striata*⁵⁰. Thus sperm investment in individual copulations can be potentially influenced by both seasonal and circadian patterns.

(ii) Social status

Social hierarchy results in an unequal distribution of contested resources, such as food,

territory, and mates. In polygynyandrous species, socially dominant males have been found to father more offspring in the population^{51,52}. Male social status affects strategic ejaculate allocation in two ways. Firstly, dominant males and subordinate males face a different level of sperm competition. Dominant males have disproportionally more mating opportunities, are more likely to guard females from remating and disrupt the mating attempts of subordinate males from mating, and face less sperm competition risk⁵³⁻⁵⁶. Subordinate males often mate with a recently mated female and thus face a higher risk of sperm competition⁵⁷⁻⁵⁹. Males are sensitive to the risk of sperm competition. For instance, Bretman et al. (2010) found that the absolute length of exposure to rival males determined male strategies in *D. melanogaster*⁶⁰. The longer the males were exposed to rivals prior to mating, the longer their subsequent mating duration, resulting in increased transfer of seminal fluid accessory gland proteins, which poses direct effects on postmating responses in females, and, ultimately, increased fertilisation success⁶⁰.

Secondly, dominant males have a better mating prospect and need to carefully balance ejaculate investment in the current mating and that in the potential future mating²⁵. For subordinate males on the other hand, future mating opportunities are less certain and scarce. Therefore, subordinate males are predicted to have invested more or higher quality sperm in an ejaculate in the current female than dominant and because they are less constrained by sperm depletion.

Empirical evidence supports the prediction. In the Arctic charr, *Salvelinus alpinus*, subordinates showed higher sperm production and higher sperm velocity than dominant males⁶¹. For example in salmon, *Salmo salar*, sneaking precocial parr faces sperm competition in every mating while the guarders experience a much less sperm competition intensity. Subordinate males have relatively bigger testes and denser ejaculates⁵⁸. In an externally fertilising fish, chinook salmon, *Oncorhynchus tshawytscha*, males can upregulate sperm velocity when their social status changed

from dominant to subordinate ⁶². In the grass goby, *Zosterisessor ophiocephalus*, sneakers (subordinate males) have sperm of higher swimming speeds and greater viability than those of territorial males (dominant males) ⁵⁹.

(iii) Female quality

For a long time, it was assumed that there is a considerable variance among males, which serves as the raw materials of sexual selection. However, biologists realised that the females can also vary in their reproductive quality. Variation in the quality of available females is a fundamental requirement for male mate choice, the greater the variation, the larger the potential benefits ⁶³. Females can vary in the quality of their reproductive output, both in terms of the number of eggs and nutrients allocated to each egg ⁶⁴. Female's body weight, body condition, age and sexual ornaments are often good indicators of female reproductive quality.

Female body size, social status, age and body conditions/ornaments are related to female reproductive quality. Significant variation in body size across females of many taxa is often positively associated with fecundity ⁶⁵. A meta-analysis across 68 insect species revealed that each 1% increase in dry body predicts a 0.95% increase in median fecundity ⁶⁶. During the breeding season, females have a higher demand for nutrition because of high reproduction costs comparing to males. It was found that hens of high social status have access to more food, matured earlier and had a larger body size than the subordinate ones ⁶⁷. Age is also found to be a strong effector of female fecundity. For example, the majority of bird species exhibit age-dependent reproduction. Commonly, inexperienced breeders or young birds perform at a slower and lower rate than older and experienced ones. The most common form is the individuals show a steady increase of fecundity and peak at middle age. Then the fecundity decreases towards the end of their lifespan ⁶⁸. Female body condition, like feather condition, can reflect the female's control over food. Feathers are susceptible to damage. Feathers that are rich in melanin are more resistant to wear. So the feather condition can reflect the

birds' ability to eat melanin-rich food and signal the mate quality ⁶⁹.

Males are also expected to respond to variation in female quality. Honest signals reflect female reproductive quality and enable males to discriminate, and theory predicts that males should allocate more ejaculate in higher-quality females ⁷⁰. For example, female body size in some species correlates with fecundity. Thus males prefer large or heavy females (i.e., in amphibian ⁷¹, bird ⁷², fish ⁷² and insects ⁷³). Theory predicts that males should allocate more ejaculate in higher-quality females ⁷⁰. Male can also adjust their mating decision based on the sexual ornamentations of females. In rock sparrows, *Petronia petronia*, the effort male put into defending the nest was significantly related to female breast patch size, which is an indicator for female quality ⁷⁴, suggesting that males can assess individual variation in female quality based on sexual ornamentation and adjust parental investment decisions in favour of more ornamented female qualities ⁷⁴.

(iv) Female novelty/availability

Males are also expected to adjust their ejaculate expenditure strategies according to female availability. Males in many species prefer to mate with a sexually novel female instead of a female with whom they previously mated ⁷⁵. This response is mediated by a behavioural mechanism known as the Coolidge effect. The Coolidge effect suggests that males have the inclination of having more mates and they can reserve some sperm for a future mate. The Coolidge effect exists in many species. The sexual interest of the male salamander, *Desmognathus ochrophaeus*, decreases over successive encounters with a female ⁷⁶. Studies provided direct evidence for higher propensity to copulation when a new partner is encountered, in a simultaneous hermaphrodite pond snail, *Lymnaea stagnalis* ⁷⁷. Behavioural patterns consistent with the Coolidge effect have been described in males of several mammalian species, like rats, hamsters, mice, voles, cats ⁷⁵ and humans ⁷⁸. The Coolidge effect is more pronounced in species with sperm competition due to the increased demands on ejaculate expenditure. Some species,

mostly monogamous species show no Coolidge effect. The absence of a Coolidge effect has now been treated as an indicator of monogamy. For example, no Coolidge effect was found in the monogamous prairie voles, *Microtus ochrogaster*⁷⁹.

The Coolidge effect might also differ according to the male's social status. The European bitterling, *Rhodeus amarus*, is an externally fertilising species. Dominant males invested more sperm in the novel females, while subordinate males were not influenced by female's novelty. This suggests males can allocate sperm strategically⁸⁰. This suggests that dominant males have more mating opportunities and tend to reserve more sperm for future matings while subordinate males use most of their sperm in current mating because future mating prospect is dim and there is a passive loss of sperm.

Cognitive mechanism

While male ejaculate expenditure strategies are well studied, little is known about the behavioural mechanism underpinning male ejaculate expenditure decisions. Past experiences could serve as a good cue of current decision-making. The cognitive mechanisms of female choice have been studied extensively. For instance, in the three-spined sticklebacks, *Gasterosteus aculeatus*, females prefer more brightly coloured males. A male is rated higher by the female when preceded by a duller male than by a brighter one and vice versa⁸¹. However, the cognitive mechanisms of male choice have rarely been studied.

There is some evidence that experience can influence current mating and ejaculate expending decisions. In *D. melanogaster*, males prefer to mate with the type of females with whom the males mated in the past. If males mated with a large female in the first trial, they would prefer to still mate with a large female in the second trial. However, if males mated with a small female in the first trial, they would prefer to mate with a small female⁸². Similarly, though male *D. melanogaster* usually courts virgin females more

vigorously than fertilised females, males perform very little courtship to a virgin female if he has previously been exposed to a fertilised female ⁸³⁻⁸⁵. Past mating experience therefore can carry over to influence current mating decisions.

Males may even learn from past experience, models predict that, compared with indiscriminate courtship, selective courtship based on previous experience may have advantages and incur higher reproductive success ⁸⁶. For example, males will not get viable offspring by mating with a heterospecific female and they need to learn to avoid heterospecific females in the future. Empirical studies of *Drosophila. persimilis* showed that males whose courtship was previously rejected by heterospecific females before, courted heterospecific females less than inexperienced males ⁸⁷.

In this thesis, I investigated less well-known aspects of ejaculate expenditure behaviour in male red junglefowl, *Gallus gallus*.

Red junglefowl

The red junglefowl belongs to the Phasianidae family ⁸⁸ and is the main ancestor of the domestic chicken ⁸⁹. The mating system of red junglefowl is polygynandrous, which leads to sperm competition. The male red junglefowl can produce a large number of sperm in ejaculates and mate multiply within a short time period, allowing for a detailed study of behavioural processes down to the level of gametes. Males and females are sexually dimorphic. Males are larger and more ornamented both with colourful plumage and with fleshy large ornaments, i.e. the comb and wattles on the head, whereas females are more cryptically coloured and have smaller combs. In the red junglefowl, *Gallus gallus*, the expression of a fleshy ornament, the comb, signals female fecundity and reproductive investment ⁵⁴. Accordingly, several studies of red junglefowl and domestic fowl, *G. domesticus*, have shown that when presented simultaneously with females of different comb sizes, socially dominant males preferentially invest in larger-combed females ^{90 44}. Females that have a larger comb

obtain more sperm from males ⁹¹.

We studied a population of red junglefowl in Oxford. Red junglefowl in Oxford is a seasonal breeder whose breeding season typically lasts from late spring to late summer.

Red junglefowl lives in socially structured groups consisting of several males and females ⁹². In natural conditions, males form dominance hierarchies, and social status mediates access to females, placing dominant and subordinate males in favored and disfavored mating roles, respectively ⁹³. Dominant males have higher copulation success and face a lower risk of sperm competition than subordinate males, however females frequently copulate with multiple males, generating high levels of sperm competition ⁹³. Relative sperm number and fertilising efficiency of an ejaculate are the primary determinants of fertilisation success ³⁰. The polygynyandrous nature of the breeding system and the high frequency with which males can copulate, can lead to sperm depletion, and males, especially dominant individuals, are economical with their gametes, adjusting the number of sperm they ejaculate according to their social status ³³ (but see ²⁴). Dominant males typically monopolise access to females and interrupt the mating attempts of subordinate males. Females also prefer dominant males by preferentially soliciting mating from them and through reduced resistance towards to the mating attempts of the dominant male ⁹⁴. To compete with dominant males, subordinate males have to invest more sperm or higher quality sperm in each copulation to compensate for the disadvantaged social role as found in several studies ^{24,55}.

Male red junglefowl also showed a heightened sexual interest towards a sexually novel female. Male invests more sperm and a different set of seminal fluid proteins when exposed to a novel female after successive mating with the familiar female ⁹⁵.

Aim

The overarching goal of my thesis is to study mechanisms and patterns of ejaculate

expenditure behaviour in the red junglefowl.

My thesis has four specific aims.

1. To characterise seasonal and circadian patterns of ejaculate expenditure.
2. To establish the causal relationship between male social status and ejaculate expenditure.
3. To investigate the role of previous experience in patterns of male ejaculate expenditure in relation to female quality.
4. To confirm the Coolidge effect in red junglefowl and explore mechanisms underpinning male ejaculate expenditure in sexually novel females.

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Chapter 2. Seasonal and Circadian Patterns of Male Ejaculate Expenditure

Abstract

Reproduction often shows marked temporal patterns. Temporal patterns may thus represent important modulators or constraints in male ejaculate investment strategies under sperm competition. Little however is known about the way male ejaculate investment varies at different temporal scales. Here, we investigate seasonal and circadian patterns in male ejaculate investment in a population of red junglefowl, *Gallus gallus*, a polyandrous bird characterised by high levels of sperm competition, and in which males modulate ejaculate investment in response to socio-sexual factors. We found that male sperm investment showed both significant linear and quadratic seasonal patterns, cumulative sperm number produced in a trial, and the number of sperm invested in the first copulation, both of which peaked in the middle of the breeding season. Latency to ejaculate, however, did not show seasonality. On the other hand, the courtship rate, but not sperm numbers or ejaculation latency, displayed a circadian pattern. Collectively, these results highlight the importance of considering patterns of variation at different temporal scales when investigating male ejaculate investment behaviours.

Introduction

Reproductive behaviour is often characterised by marked temporal patterns at different scales, such as over a reproductive season (i.e., seasonal pattern) and over the course of a day (i.e., circadian pattern). Studying seasonal and circadian patterns is critical to understand the regulation, constraints and functional significance of reproductive behaviours. Temporal patterns have been extensively investigated in the context of the proximate regulation of breeding. Little however, is known about their potential influence on male ejaculate expenditure and causative factors.

Seasonal patterns in reproduction can reflect the responses of the organism to temporal changes in environmental conditions, such as food availability, temperature and precipitation, which influence the outcome of reproductive attempts and offspring performance. Due to these environmental constraints, reproduction in many organisms is typically restricted to certain times of the year¹. In temperate zones, where warmer seasons alternate with cold seasons, food availability and suitable climate is usually restricted to short, predictable windows, especially at higher latitudes. Juvenile Atlantic salmon, *Salmo salar*, can switch between diurnal and nocturnal foraging solely in response to environmental temperature, and independently of the photoperiod and the season. Above 10 °C juvenile Atlantic salmon fed predominantly during daylight, spending the night in the water column during which time in a relatively quiescent state. As the temperature dropped below 10 °C, however, they became increasingly nocturnal, hiding in refuges by day but emerging to feed at night².

Thus, seasonal patterns have been well characterised in species with temperate distribution, which typically display synchronised, predictable, population-wide reproductive cycles³. Strong seasonal patterns are also observed in tropical species that do not experience alternation between warm and cold seasons but experience other periodic climatic fluctuations, for example, rainy seasons alternated with drier seasons⁴. The resource availability hypothesis explains the functional significance of seasonal

patterns by predicting that reproduction is timed to track maximal resource availability⁴. For mammals, for example, lactation is energy-consuming and for female Assamese macaques, *Macaca assamensis*, in Thailand, the annual birth season is timed for lactation to coincide with the annual peak in food availability⁵. At a proximate level, the reproductive cycle of many organisms, especially those in temperate zones, is known to be dependent on photoperiod, which, in turn, may stimulate endocrine triggers. In the house finch, *Carpodacus mexicanus*, testicular maturation can occur when birds are exposed to only six hours of light in each experimental cycle⁶. Similarly, exposure to a long-day light regime, triggers rapid testicular growth and reproductive behaviour in male Japanese quail, *Coturnix japonica*⁷. In addition to the photoperiod signal, the timing of reproduction can also be further modulated by ambient temperature. For example, in some wild bird populations (i.e. great tits, *Parus major*), the mean laying date occurs earlier in warmer years than in colder years⁸.

In addition to seasonal patterns, reproductive behaviour can also peak at certain times of the day. Circadian (circa = around, diem = day) pattern is a daily change of a given behaviour or physiological process⁹. Circadian patterns are important in coordinating reproduction, feeding and sleeping patterns, and are thought to enhance survival and reproductive fitness, in part by optimising the timing of behavioural and physiological processes in line with fixed cycles in the environment¹⁰, i.e. by minimising competition and predation risk, avoiding poor environmental conditions, and maximising reproductive outputs, such as mate encounter rate or the probability of fertilisation¹.

While reproductive behaviour is typically modulated over seasonal and circadian time scales, few are known about the way these temporal scales influence patterns of male ejaculate expenditure. Fully flexible behavioural plasticity is essential to animals in a world full of uncertainty. Because strategies can be rapidly reversible and are potentially less 'expensive'^{11 12} than changing fixed strategies. Behavioural plasticity enables animals to respond to short-term environmental fluctuations¹³. Bretman et al.

proposed that seasonality predicts high plasticity because males have limited fertilising capacity ¹⁴. For example, in the threespine stickleback, *Gasterosteus aculeatus*, a seasonal breeder, males have flexible mating strategies at different times during the breeding season, modulated by seasonal changes in future reproductive opportunities. Males increased risk-taking when future reproductive opportunities decreased late in the breeding season ¹⁵. Seasonal and circadian effects may be particularly important in polyandrous species, where ejaculate expenditure strategies are an important aspect of male reproductive fitness.

In many species females mate with multiple males (polyandry) which generates sperm competition. Sperm competition is the process that sperm from different males compete for the same set of ova ¹⁶. Males are selected to enhance their fertilisation success by either allocating more sperm or better-quality sperm. However, sperm is not limitless and ejaculate is costly to produce. Ejaculate production may trade-off with premating sexual traits ^{17 18-21} or non-reproduction functions mating ^{22 23 24 25 26 27,28}. Optimal sperm allocation models under sperm competition adopt an evolutionarily stable strategy (ESS) approach developed by Smith ²⁹. This approach assumes that the outcome of one player in the game depends on the rival's strategy. As in sperm competition, the sperm allocation strategies of one male depends on the sperm allocation strategies of other males. Optimal sperm allocation models assume i) males have a limited amount of sperm, so increasing each ejaculate will result in a reduction in the total number of matings.; ii) males are able to vary the number of sperm. Theory predicts males are able to adjust sperm investment under different contexts ¹⁶.

Despite extensive research into seasonal and circadian patterns in animal behaviour, few are known about their potential influence in male ejaculate expenditure. Marked changes in semen characteristics over the course of a reproductive season have been well established in a number of species ³⁰. For example, in rams, *Ovis aries*, semen volume and sperm count showed both a linear and quadratic effects over the course of

the season from April to August³¹. In hatchery-reared lake trout, *Salvelinus namaycush*, sperm swimming velocity followed a negative quadratic function over the breeding season from October to December, whereas sperm longevity and density had a positive quadratic pattern³². In simmental bulls, *Bos taurus*, higher percentages of major sperm defects were found in the summer than in the winter³³. In stallion, *Equus caballus*, semen volume was greatest in the summer but both the concentration and the total number of spermatozoa per ejaculate were highest in the autumn³⁴. According to a study on humans, sperm concentration, mean percentage of normal sperm were significantly higher in winter³⁵. In other species, however, seasonal patterns are less conspicuous. For example, in swamp buffalos, *Bubalus bubalis*, in Thailand, the season does not affect semen sperm output or sperm viability³⁶.

A number of species also showed marked circadian patterns in male mating propensity and semen characteristics. In Japanese quail, *Coturnix coturnix japonica*, high temperature and high humidity can have adverse effects on sperm quality³⁷. Lekking females sharp-tailed grouse, *Tympanuchus phasianellus*, have been found to mate preferentially around sunrise, during which time, the body and testicular temperature of lekking males are cooler compared with 150 minutes after sunrise³⁸. Diurnal variation in semen quality has been investigated in humans. One study has found that across 54 semen donors, the number of spermatozoa with progressive linear motility was higher in the afternoon than in the morning³⁹. However, another study has found that the human sperm concentration, total sperm count and the proportion of normal morphology are highest in the morning⁴⁰. These studies suggest that semen production and mating behaviour can change over the course of the reproductive season and over the course of the day. Therefore, these temporal scales can potentially influence patterns of male ejaculate expenditure.

The overarching goal of this chapter is to study the seasonal and circadian patterns of ejaculate expenditure in a seasonal, polyandrous bird, the red junglefowl, *Gallus gallus*.

The red junglefowl, belongs to the Phasianidae family ⁴¹ and is the main ancient ancestor of the domestic chicken, *Gallus domesticus* ⁴². The geographical distribution of wild red junglefowl extends from the northeast and central India, to the extreme South of China, and Thailand ⁴¹. The mating system of red junglefowl is polygynandrous, where both males and females typically mate with multiple mates. Average polyandry has a direct effect on sexual selection on males, largely by negating the strength of precopulatory sexual selection. More polygynous (mate with multiple females) males tend to mate with more polyandrous (mate with multiple males) females, drastically decreases the intensity of precopulatory sexual selection on male mating success ⁴³. Sperm competition leads to patterns of differential ejaculate expenditure by male junglefowl ¹⁶. For example, males invested more sperm to higher-quality females, novel females and adjusting their investment strategies based on their own social status ⁴⁴.

Seasonal patterns in sperm production were recently investigated in a four-year study of 1148 semen samples from a captive red junglefowl population in Pakistan ⁴⁵. This study indicated that while males of this population produced semen throughout the year, semen volume, the number of sperm produced, sperm motility, sperm plasma membrane integrity, sperm viability and acrosomal integrity were highest in spring ⁴⁶. A similar pattern is observed in domestic male fowl, *G. domesticus*, which produces the greatest number of sperm in spring and sperm production peaks in April and May⁴⁶.

In addition to these broad seasonal patterns in sperm production, there is also some evidence for circadian patterns. Male fowl showed a diurnal rhythm of testicular temperature and spermatogenesis. Spermatogenesis rate is inversely correlated with the body and testicular temperatures and spermatogenesis is fastest when body and testicular temperatures are the lowest ^{47,48}. Morning and afternoon temperatures are generally lower than that in midday and spermatogenesis might be more efficient during these times. Parker et al. found that in a population of domestic fowl, males produced

more sperm in the afternoon during voluntary mating than in the morning but this difference was not significant⁴⁹. Lake et al. found that in domestic fowl, males had significantly more extragonadal semen more sperm in the afternoon than in the noon when semen samples were collected manually⁵⁰. This previous work however, is largely based on the manual sampling of males and provides little information about the way male investment behaviour in natural ejaculates changes over the breeding season and over the course of the day.

Aims

In this chapter we develop an experimental approach to investigate seasonal and circadian plasticity in ejaculate investment behaviour in male red junglefowl. Specifically, the chapter aims to test whether male investment in natural ejaculates displays (i) seasonal and (ii) circadian patterns in a population of red junglefowl.

Materials and methods

We studied a captive population of red junglefowl housed at the John Krebs's Field Station (Oxfordshire). The population was founded in 2006 from another captive populations from Sweden. The breeding season of red junglefowl in Oxford starts in late March and ends in September, although sometimes the breeding season extends to October. We studied adult males that were sexually rested for at least two days to guarantee the maximal replenishment of their extragonadal sperm reserves ^{51,52}. All sampling of birds was conducted in accordance with approved experimental protocols under Home Office license (PPL P50402706).

General methodology: Collection of natural ejaculates through controlled copulations

We collected natural ejaculates using a well-established protocol based on the experimental exposure of a male to a female fitted with a harness covering her cloaca to prevent insemination and facilitate ejaculate collection ⁵³. We manually held a harnessed female for one minute facing the focal male in order to enable the male to inspect her and familiarise with her. Courtship rate was measured as the number of waltzes in one minute before putting the female in a soliciting position. A waltz is a typical behaviour where the male lowers one wing and circles the female and represents one of the main components of courtship in this species ⁵⁴. The female was then turned around and held in a sexually soliciting position, placed on a plastic substrate. We recorded the time from presenting the female in a soliciting position to ejaculate, as this measure may reflect male propensity to invest in a mating (i.e. ⁵⁵). We used twenty sexually mature females. Females varied in their comb size. Female reproductive quality covaries with the size of the female comb, a fleshy ornament. Males tend to invest more sperm in large-combed female (see Chapter 4 results). We measured their comb length using a ruler and measured their comb size using image J (v1.51w) adjusting the scale against the ruler. Twenty females were used, six large-comb (Appendix Fig. A1. mean $\pm$ s.e.: $3.80 \pm 0.66 \text{ cm}^2$), six small-comb ($1.47 \pm 0.18 \text{ cm}^2$) and eight intermediate-comb ($2.10 \pm 0.07 \text{ cm}^2$) females.

After each copulation, we collected ejaculates into an Eppendorf tube using a Gilson pipette. Within three hours upon ejaculate collection, sperm numbers were estimated by diluting 2.5 μ l of semen into 197.5 μ l of PBS and transferred 70 μ l of the mixture to a spectrophotometer (Scientific Laboratory Supplies, UV 1101) set at 595nm to measure the concentration of sperm within the ejaculate through the light absorbance. We measured this twice for each ejaculate and computed the arithmetic mean. We then estimated sperm numbers through a standard curve as a measure of light absorbance^{56,57}. We used standard curve developed for this population ($R^2 = 0.98$ ⁵⁸). To control for marginal daily variation in absorbance, for each day we measured light absorbance of PBS-only samples and used this absorbance to force the standard curve through the origin.

Within three hours of collecting the ejaculate, the ejaculate in the Eppendorf was briefly spun down in a Mikro 200 R Hettish centrifuge, by pressing the “impulse” key for a few seconds and stopped it by releasing the key. Then we measured volume using a Gilson pipette.

Seasonal patterns

We investigated whether patterns of ejaculate expenditure changed through seasons by combining samples collected across three experiments conducted during the breeding season from April to August 2018. The first experiment was done from April to July and is presented in chapter 5. The second experiment was done from June to July, and the third experiment was done from July to August. These latter two experiments are presented in chapter 4. All three experiments followed a similar trial protocol. In each trial a male was given the opportunity to mate up to six times within 60 minutes either in the morning or evening within 60 minutes. If the males did not mate for 10 (third experiment) or 15 (first and second experiment) minutes, we terminated the trial. Each male had a trial every two days. All males were kept in individual pens where they had

access to food and water at all times for 10 days (second experiment), 16 days (third experiment), or 30 (The first experiment) days. Prior to the experiments, males were kept in single-sex pens of around 12 birds. All males used in the experiments were non-virgins and they were sexually rested for at least 48 hours. For the first experiment, males were kept visually isolated, but they can hear each other. For the second and third experiment, males were not visually isolated. At the end of an experiment, males were returned to their original pens. In total, thirty-three males were used but only thirty-two males were included in the dataset, excluding one male that did not produce ejaculate for all trials. Males were removed from the experiment after failing to ejaculate on two successive trials. Some males were used in multiple experiments.

In the first experiment, in some trials the female was replaced with a new female after the third mating opportunity while in other trials the same female was retained for all 6 mating opportunities. All females were of intermediate-comb size (Appendix Fig A1). In the second experiment, all males had only one female per trial, but females varied in comb size across trials, and were categorised as large- intermediate- or small-combed (Appendix Fig A1). In the third experiment, all males had one large-combed and one small-combed female sequentially (Appendix Fig A1).

Circadian pattern

Eighteen males were selected for the experiment which was conducted in September 2018 and June 2019. Three males did not produce ejaculate for all trials and were excluded from the data analysis. Fifteen males were included in the dataset. They were kept in individual cages for five days where they had access to food and water at all times. The ejaculate was collected three times for each male, in the morning 8.30-9.00 am, in the midday 12.45-1.15pm, and in the afternoon 5.00-5.30 pm. We terminated a trial if males did not mate within 10 minutes. We measured ejaculate volume, sperm concentration, and courtship rate and recorded the time to ejaculation.

Statistical analysis

We conducted the analyses using the statistical software R with the lme4, stats, lsmeans, ggplot2, dplyr, car packages (R version 3.6.2).

Seasonal patterns

We tested for seasonal patterns in: (i) the cumulative number of sperm produced by a male over the six mating opportunities of a trial, (ii) the number of sperm produced by a male in the first mating of a trial, and (iii) the time elapsed to first ejaculate in a trial.

(i) *Standardised cumulative sperm*

To reduce between-male variation in the cumulative number of sperm produced by a male in a trial, we standardised cumulative sperm number in a trial by dividing the number of sperm of a male's ejaculate against the largest cumulative sperm number produced in the season by that male. Therefore, the standardised sperm ranges from 0 to 1. Similarly, day of a trial was expressed as a fraction of the entire study period (i.e. 144 days), with the first day of trials = 1/144, and the last day as day 1. The first day is 9th April 2018. We first conducted a generalised linear mixed model with standardised cumulative sperm numbers as the response variable. The response variable had a right-skewed residual plot and was therefore square root- transformed. Relative day and relative day² were entered as fixed effects to test for linear and quadratic temporal patterns. We also included trial number (from 1 to up to 20 for individual males) and male age as fixed effects. Male age affects male probability of ejaculation sperm and ejaculate parameters in fowl ⁵⁹. Because in some trials males were exposed to two females sequentially and to only one female in others, we included the number of females in a trial (one or two) as a random effect. Similarly, female comb size varied within and across trials, and we included female comb size categories (large, intermediate, and small) as a random effect. Finally, we included male ID as a random effect. We used drop1 function (Chisq) to drop out one term each time and tested the significance of each term by comparing the model with and without that term using

anova type II analysis. Chi-square distributions have only one tail and therefore the significance values were one-tailed.

```
lmer(sqrt(Standardised cumulative)~relativeday+relativeday^2+age+Trial+(1|female number)+(1|Male ID)+(1|Female comb size category)
```

Because the distribution of standardised cumulative sperm numbers was zero-inflated, we followed up this approach by investigating whether seasonal patterns were driven by the probability of ejaculation sperm or by changes in sperm numbers, with a hurdle model, which is a two-part model that specifies one model for zero counts (i.e. the probability of ejaculation) and another model for non-zero counts (i.e. only for trials in which sperm were produced). We therefore fitted a generalised linear mixed model with a binomial error structure for the probability of ejaculation. The response variable was whether the male produced sperm in a trial (1) or not (0). Relative day, relative day², male age and trial number were entered as fixed effects to test for linear and quadratic temporal effects. Finally, we included male and female ID as random effects. For each copulation, one female was used. So we used female ID instead of female number and female comb size categories as the random effect. We used drop1 function (Chisq) to drop out one term each time and tested the significance of each term by comparing the model with and without that term using anova type II analysis.

```
glmer (Produce sperm or not ~ relativeday + relativeday^2+ age + Trial + (1 | Male.ID)+(1|Female.ID), family=binomial)
```

We then focused on non-zero cumulative sperm numbers and fitted a linear mixed model with non-zero square root-transformed standardised cumulative sperm numbers as follows:

```
lmer(sqrt(Standardised Cumulative) ~ relativeday + relativeday^2 +age+Trial+(1 | Male.ID) + (1 | female.no) + (1 | Female.quality)
```

We used drop1 function (Chisq) to drop out one term each time and tested the significance of each term by comparing the model with and without that term using anova type II analysis.

(ii) *Standardised sperm in the first copulation*

We analysed variation in the number of sperm produced in the first mating of each trial using a similar approach. We standardised sperm numbers against the largest first ejaculate produced by a male across the season. We started with a generalised linear mixed model. In this case number of females and female comb size categories were replaced by female ID as a random effect as thus:

```
lmer(sqrt(Standardised sperm number) ~ relativeday + relativeday^2 + age + Trial
+(1|Male.ID) +(1|Female.ID)
```

We used drop1 function (Chisq) to drop out one term each time and tested the significance of each term by comparing the model with and without that term using anova type II analysis.

The hurdle model is

```
Glmer (logical ~ relativeday + relativeday^2+age +Trial + (1 | Male.ID) +(1|Female.ID),
family=binomial)
lmer(sqrt(Standardised sperm number) ~ relativeday + relativeday^2+ age + Trial + (1
| Male.ID) +(1|Female.ID)
```

Finally, we analysed the time to ejaculate at the first mating opportunity of a trial. We excluded trials where the male did not mate. We computed the natural logarithm-transformed (Time(s) +1) because the residual plot was right-skewed. We used time + 1 because some values were zero as males ejaculated as soon as the trial started. We fitted a linear mixed model with the natural logarithm-transformed (Time + 1) as the response variable, relative day, relative day², trial number and male age as fixed effects, female ID and male ID as random effects, as follows:

```
lmer(log(Time + 1) ~ relativeday + relativeday^2 + age+ Trial + (1 | Male.ID)
+(1|Female.ID))
```

We used drop1 function (Chisq) to drop out one term each time and tested the significance of each term by comparing the model with and without that term using anova type II analysis.

Circadian patterns

We tested for circadian patterns by investigating variation in: (i) standardised sperm numbers, (ii) male latency to ejaculate, and (iii) rate of male courtship of a female. We fitted a generalised linear mixed model for each response variable ((i)-(iii)). The number of sperm produced in an ejaculate was standardised against the largest ejaculate produced by each male in the experiment as above, and square root-transformed. The standardised sperm number ranges from 0 to 1. Similarly, the latency to ejaculate (in seconds) was log-transformed. Because no male ejaculated within the first minute of being exposed to the female, time was positive. Courtship rate on the other hand, was normally distributed and was not transformed. Because courtship rate is a count data, we used Poisson distribution first but the model was too overdispersed. So we chose the linear mixed model instead. For each response variable we included time of day (morning, noon, afternoon), year, trial (1-3) and time of day by year interaction. Male and female ID was entered as random effects as follows. We used drop1 function (Chisq) to drop out one term each time and tested the significance of each term by comparing the model with and without that term using anova type II analysis. Chi-square distributions have only one tail and therefore the significance values were one-tailed.

```
lmer(Standardised.sperm~Time of the day*year+Trial+(1|Male.ID)+(1|Female.ID))
```

```
lmer(log(Time)~Time of the day*year+Trial+(1|Male.ID)+(1|Female.ID))
```

```
lmer(courtship~Time of the day*year+Trial+(1|Male.ID)+(1|Female.ID))
```

Both, seasonal and circadian analyses test the same general hypothesis several times.

We therefore in addition to the 5% significance threshold, controlled for the risk of a false positive due to multiple comparisons using Bonferroni corrections⁶⁰. Thus for the seasonal analysis for the standardised cumulative sperm number, the significance threshold was set at 1.67% (3 comparisons). For the seasonal analysis for the standardised sperm in the first mating, the significance threshold was set at 1.25% (4 comparisons). For the circadian analysis, the significance threshold was set at 1.67% (3 comparisons). We used an asterisk to signal the terms that were no longer significant after Bonferroni correction.

Results

Seasonal results

(i) Standardised cumulative sperm number

We found significant linear and quadratic effects of males' cumulative standardised sperm number (Table 1a, Fig. 1a, b), the probability of ejaculation (Table 1b, Fig. 1c, d), and non-zero cumulative numbers of sperm (Table 1c, Fig. 1e, f). They showed a similar pattern that peaked in the middle of the season and declined linearly over the course of the season (Fig. 1). Age does not have an effect (Table 1). Trial does not have an effect (Table 1).

Table 1. the analysis of variance (ANOVA) table

of Seasonal effects in cumulative numbers of sperm produced in a trial.

(a) full lmer on the square root-transformed cumulative standardised sperm number

Response variable	Factors	df	Chisq	P value
Sqrt (Standardised sperm number)	(day)^2	1	30.283	<0.001
	day	1	15.530	<0.001
	age	1	0.000	0.994
	Trial	1	0.066	0.798

(b) glmer on probability of ejaculation of all copulation

Response variable	Factors	df	Chisq	P value
Probability of producing ejaculates	(day)^2	1	17.858	<0.001
	day	1	9.415	0.002
	age	1	0.052	0.819
	Trial	1	1.185	0.276

(c) lmer on non-zero cumulative standardised sperm numbers

Response variable	Factors	df	Chisq	P value
Sqrt (Standardised sperm number)	(day)^2	1	24.518	<0.001
	day	1	14.739	<0.001
	age	1	0.092	0.762
	Trial	1	0.847	0.357

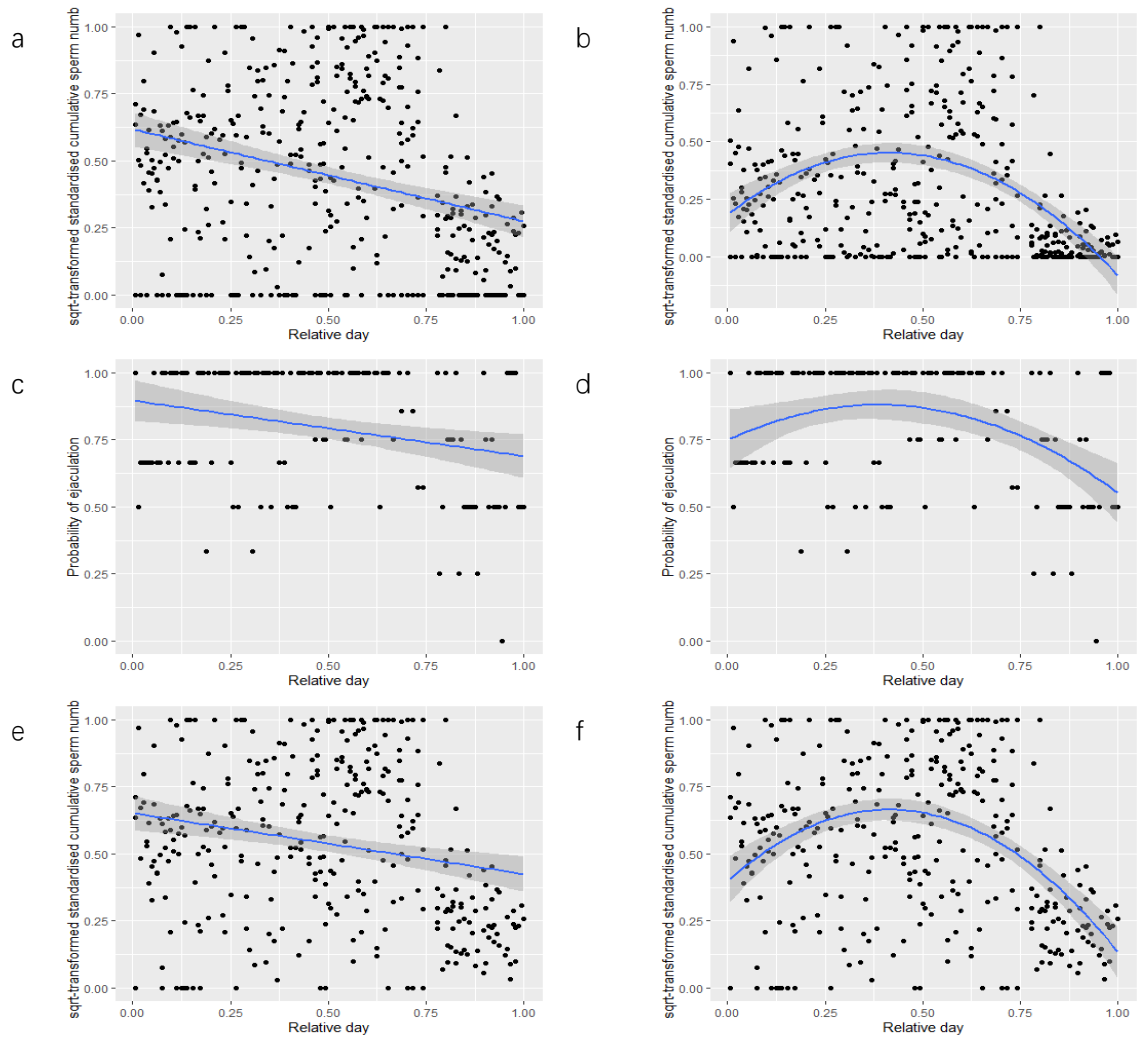


Figure 1. Linear (a) and quadratic (b) regressions of square root-transformed cumulative standardised sperm. Sperm number is standardised against the highest cumulative sperm number that the male produced during the whole breeding season. Linear (c) the quadratic (d) regressions of the probability of ejaculation. Linear (e) and quadratic (f) regression of square root-transformed cumulative standardised sperm excluding zeros (i.e. cases where no sperm were ejaculated). The X axis represents the relative day. Days are standardised as a fraction of the entire study period (i.e. 144 days) and the last day as day 1.

(ii) *Standardised sperm number of the first copulation*

Similarly, we found significant linear and quadratic effects of males' standardised sperm number for the first copulation (Table 2a, Fig. 2a, b), the probability of ejaculation (Table 2b, Fig. 2c, d), and the non-zero standardised sperm number (Table 2c, Fig. 2e, f). The seasonal effects were both driven by a sudden stop of producing ejaculates (Table 2b, Fig. 2c, d) and a gradual reduction in sperm number (Table 2c, Fig. 2e, f). The linear and quadratic terms of the latency to ejaculate are not significant after Bonferroni correction (Table 2d, Fig. 2g, h). Age does not have an effect (Table 2). The trial does not have an effect (Table 2).

Table 2. the analysis of variance (ANOVA) table

of Seasonal patterns in the number of sperm ejaculated at the first copulation

(a) *full lmer on the square root-transformed standardised sperm number at the first copulation*

Response variable	Factors	df	Chisq	P value
Sqrt (Standardised sperm number)	(day)^2	1	27.049	<0.001
	day	1	16.959	<0.001
	age	1	0.134	0.714
	Trial	1	2.731	0.098

(b) *glmer on probability of ejaculation at the first copulation*

Response variable	Factors	df	Chisq	P value
Probability of producing ejaculates	(day)^2	1	16.234	<0.001
	day	1	8.362	0.004
	age	1	0.078	0.779
	Trial	1	0.878	0.349

(c) *lmer* on the square root-transformed non-zero standardised sperm numbers at the first copulation

Response variable	Factors	df	Chisq	P value
Sqrt (Standardised sperm number)	(day)^2	1	9.057	0.003
	day	1	9.108	0.003
	age	1	0.030	0.863
	Trial	1	3.251	0.071

(d) *lmer* on latency to ejaculate at the first copulation

Response variable	Factors	df	Chisq	P value
Log (Time + 1)	(day)^2	1	6.128	0.013*
	day	1	4.189	0.041*
	age	1	0.931	0.335
	Trial	1	0.034	0.853

*no longer significant after Bonferroni correction (new significance threshold 1.25%)

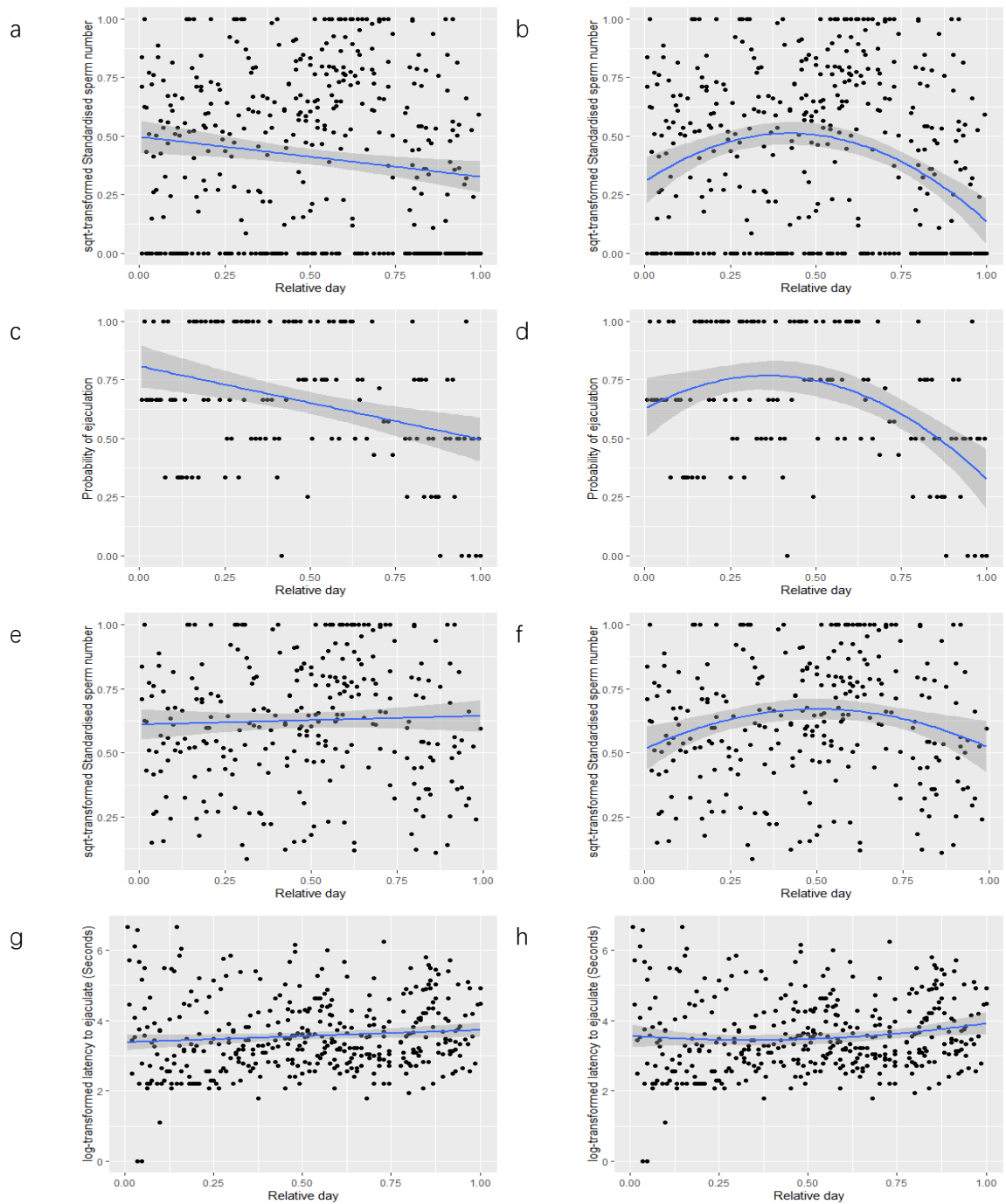


Figure 2. Linear regression (a) and (b) quadratic regression of square root-transformed standardised sperm in the first copulation. Sperm number is standardised against the highest sperm number in the first copulation that male produced in the whole breeding season. Linear (c) and quadratic (d) regressions of the probability of ejaculation in the first copulation. Linear (e) and quadratic (f) regressions of square root-transformed standardised sperm number excluding zeros in the first copulation. Linear (g) and quadratic (h) regressions of the natural log-transformed latency to ejaculate in the first

copulation. The x axis represents the relative day. Days are standardised as a fraction of the entire study period (i.e. 144 days) and the last day as day 1.

Circadian

There were no significant differences in sperm allocation and latency to ejaculate at different times of the day (Table 3a-b, Fig. 3a-b). As for the latency to ejaculate, males in the second year were consistently significantly faster to ejaculate than males in the first year (Table 3b, Fig. 3b). As for the courtship rate, there was a significant Time of the day by year interaction effect (Table 3c, Fig. 3c). In year one, males courted females more frequently during noon than in year two. In year 2, males courted females more often in the morning than in the noon (Table 3c). Trial was not significant after a post-hoc test (Table 3c).

Table 3. the analysis of variance (ANOVA) table

(a) lmer on standardised sperm

Response variable	Factors	df	Chisq	P value
Standardised sperm	Time of the day : year	2	4.624	0.099
	Time of the day	2	2.94	0.230
	Trial	2	2.544	0.280
	Year	1	0.033	0.856

(b) lmer on latency to ejaculate

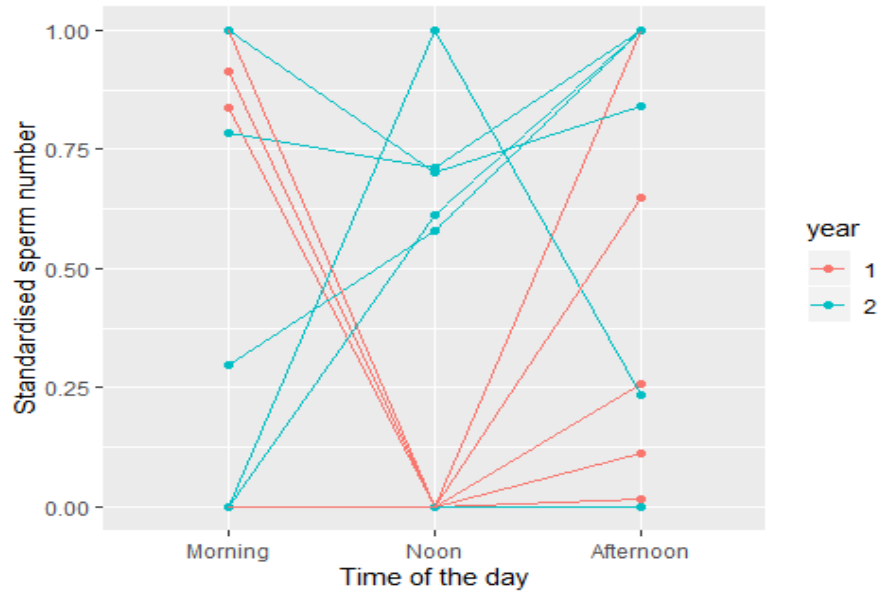
Response variable	Factors	df	Chisq	P value
Log-transformed time	Time of the day : year	2	5.969	0.051
	Trial	2	2.259	0.323
	Time of the day	2	3.252	0.197
	Year	1	9.248	0.002

(c) lmer on courtship rate

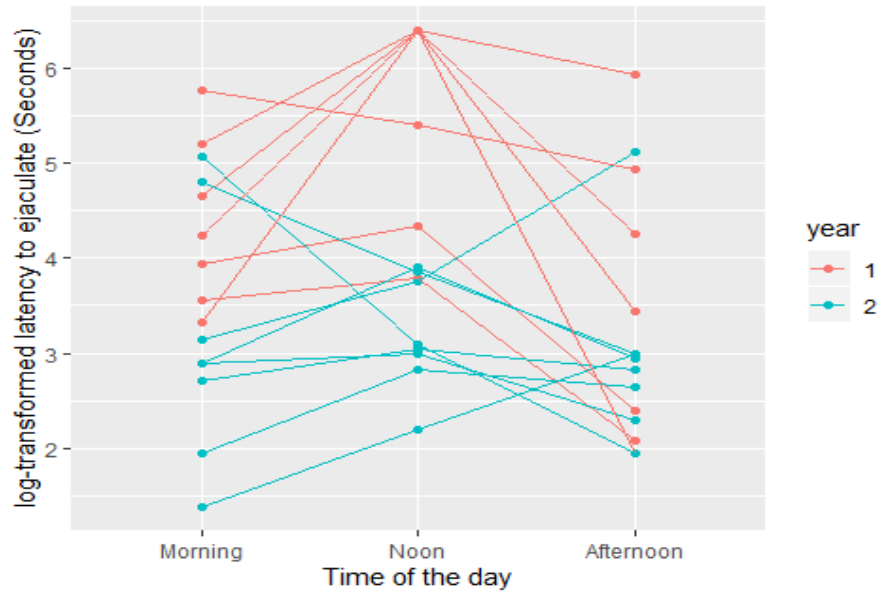
Response variable	Factors	df	Chisq	P value
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Courtship rate	Time of the day : year	2	17.324	<0.001
	Trial	2	8.550	0.014
	Time of the day	2	3.317	0.190
	Year	1	1.787	0.181
Post-hoc: Noon,1 - Noon,2 P=0.045				
Morning,2 - Noon,2 P=0.024				

a



b



c

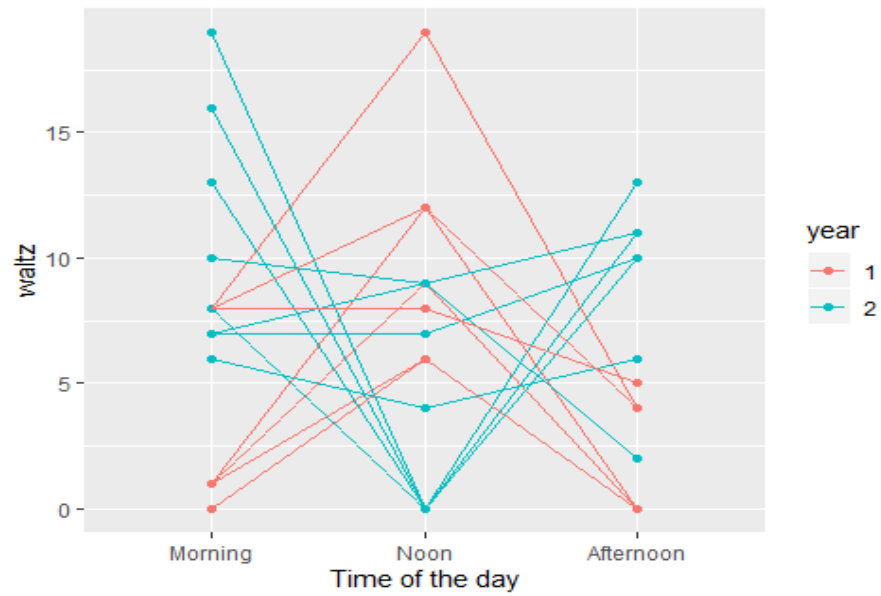


Figure 3. (a) the standardised sperm investment in the morning, noon and afternoon over the first (red) and second (blue) year of study. (b) the natural log-transformed latency to ejaculate (in seconds) in the morning, noon and afternoon. (c) Courtship rate in the morning, noon and afternoon.

Discussion

In this chapter we investigated seasonal and circadian patterns in different aspects of ejaculate expenditure and mating propensity in a polyandrous bird species, the red junglefowl.

We found a significant seasonal pattern in the cumulative sperm investment, first copulation sperm investment but no significant seasonal pattern in the latency to ejaculate. Firstly, the sperm investment peaked in June and was lowest in early April and August. Secondly, the linear regression of the sperm investment showed a downward trend, meaning that overall males invested fewer sperm in the end of the breeding season than the start. Thirdly, the latency to ejaculate followed an opposite albeit non-significant trend. We found no evidence for circadian patterns, although we observed differences in circadian patterns of courtship rate over the two years of study.

Seasonal

Linear and quadratic effects of day indicate male junglefowl gradually increased ejaculate expenditure over spring, with a peak in late June – early July, and a subsequent decline in August. This pattern is consistent with a male strategy to tailor reproductive investment to the availability of reproductive opportunities as described in this seasonal breeder (breeding season April to June) ⁶¹. Males may be constrained by the ability to produce sperm. Gametogenesis in birds is tightly regulated by photoperiod. In the house finch, *Carpodacus mexicanus*, testicular maturation can occur when birds are exposed to only six hour of light in each cycle ⁶. Once Japanese quail, *Coturnix japonica*, are transferred to long-day conditions, their testes grow rapidly, and start to reproduce. Similarly, in red junglefowl reproduction is controlled by photoperiod and exposure to 20-hour light and 4-hour dark daylight regime quickly triggers gonadal development ⁶². Birds kept in 20L (light) :4D (dark) conditions showed a marked difference in both the testicular and ovarian weights compared to males kept in 6L:18D conditions. The fact that ejaculate expenditure peaks in late June to early July, coincides with the time

shortly after the annual peak in day length, suggesting that male ejaculate expenditure behaviour may closely match gonadal development and spermatogenesis rates.

As for the downward linear regression, the pattern could be explained by the experimental methodology. (a) the first experiment was conducted at the start of the breeding season, we only used males that can ejaculate in the pilot test (one successful ejaculation with a randomly selected female). Those males who did not start breeding were excluded. Towards the end of the breeding season, males stopped ejaculation for consecutive two trials and the number was entered as zeros. (b) we started the experiment in early April. However, some males may have started reproducing early in March. Therefore, we may have missed part of the early seasonal increase in sperm investment.

The patterns of cumulative sperm investment over the trial and in the ejaculate of the first copulation were similar. This suggests that males increased their sperm allocation both by increasing the number of sperm in each ejaculate and by increasing the number of ejaculates. The latency to ejaculate showed no seasonal effect, suggesting males were still sexually interested in females but were constrained by the limited production of sperm. The probability of ejaculation showed a more obvious seasonal pattern than the non-zero sperm number, suggesting the seasonal effects were mainly driven by the ability of the male to ejaculate successfully. In birds, ejaculation happens when semen is expelled out by cloaca contraction ⁶³. I proposed two hypotheses to explain these results. The first hypothesis is that semen volume needs to exceed a certain threshold before it can be ejected. Red junglefowl store semen in the soft and long vas deferens (VD). If the VD is full, it is easy to expel the contents. However, when it is not fully occupied, semen is less likely to be expelled. Males may be constrained by an internal semen volume threshold which determines whether they will ejaculate or not in a copulation. When the extragonadal semen reserve is below the threshold, they will not ejaculate. The second hypothesis is that males may not be able to generate sufficient

powerful cloacal contractions when the sexual stimulus is not strong enough, thus resulting in failed ejaculation ⁶⁴.

Circadian

For circadian patterns, we did not find a significant circadian pattern in terms of sperm number. However, the latency to ejaculate was significantly higher in year 1, suggesting that they were less sexually interested in the year 1 and the courtship rate was significantly higher in the morning than in the midday of year 2 and between years, males courted females significantly more in the midday of year 1 than in the midday of year 2.

We compared our experiment with two previous studies of circadian patterns in ejaculate size in male domestic fowl. Lake⁵⁰ found that sperm number was significantly greater in the morning and afternoon than that in the midday. Parker⁴⁹ found that no significant difference between morning and afternoon. There were several major differences in our experiments summarised in the table below.

	Our experiment	Lake's experiment ⁵⁰	Parker's experiment ⁴⁹
Semen collection	Controlled copulation with a harness attached to females	Abdominal massage	Mating with a collector attached to males
Month	June, September	May, June	April, June
Time	8.30-9.00, 12.45-13.15, 17.00-17.30	08.30-09.30, 12.00-13.00 and 17.00-18.00	15 minutes in the morning and 15 minutes in the afternoon
Number of 1 ejaculates	1	1	Multiple
Statistical analysis of sperm allocation	Time of the day: Chisq	Time of the day: Chisq statistics	Time of the day: t statistics = 0.675,

	statistics=4.624, P=0.099	=8.238 P=0.016	p-value = 0.530*
		Post-hoc: Noon- Afternoon P=0.026*	
Species	Red junglefowl	Domestic chicken	Domestic chicken

*We re-analysed their data (See Appendix Table A1).

Firstly, Lake used a non-invasive manual method to collect semen based on abdominal massage. Abdominal massage can reflect the male's extragonadal sperm reservoirs instead of ejaculate expenditure, which may be considerably smaller than the male's entire budget ⁶⁵. Sperm release from the testis may have a circadian rhythm. In wild gypsy moth, *Lymantria dispar*, 50% of males had released sperm from the testes into the vasa deferentia 3 h before dusk ⁶⁶. For domestic fowl, *Gallus gallus domesticus*, the mitotic activity was greatest during the night which suggests that the production of spermatozoa may not be uniform during the day ⁴⁸. If male red junglefowl also has this kind of circadian of sperm release (i.e. 3 hours before dusk), they are predicted to have more sperm reserved during the evening and the morning. Due to the constant passive loss of sperm, they would store fewer sperm in the midday, which is consistent with Lake's findings. It is possible that in the midday, males stored fewer sperm but this difference may be too slight to manifest itself in the first copulation. Parker used a non-invasive glass device attached to male's cloaca for semen collection and allowed males to mate with females without controlling for behavioural interactions. For example, females could resist or disrupt the mating process. Wearing a harness may also affect male performance and semen sample often mixed with faeces⁶⁷ (Appendix Fig A2).

Secondly, Lake collected sperm in the morning, midday and afternoon while Parker only collected sperm in the morning and afternoon. Both the results of these previous studies are consistent with our results in showing that sperm numbers in the morning

and afternoon do not differ significantly. However, for the midday, we did not find any significant difference whereas Lake found that males in afternoon trials had more sperm than at noon.

Thirdly, both Lake and our experiment only had one ejaculate but Parker's experiment had multiple ejaculates and computed the cumulative sperm number in one trial. According to the previous result we found, the cumulative sperm investment and sperm investment in the first copulation were similar. Sperm number in a natural ejaculate is generally smaller than that in that expelled by abdominal massage⁶⁵.

Fourthly, we did a more comprehensive study. We also recorded the latency to ejaculate and the courtship rate. In our experiment, the time to ejaculate was significantly higher in year one (September) than in year 2 (June), suggesting males were less sexually interested in mating. We did the experiment in two breeding seasons, one in June and one in September. September represents the end of the breeding season while June represents the middle of the breeding season. However, we did not find a significant seasonal pattern of the latency to ejaculate after Bonferroni correction, which is inconsistent with what we found here. Therefore, the difference may either be a year effect (year 1 vs year 2) rather than a seasonal effect (June vs September) or that the Bonferroni correction (1.25%) is too conservative. We could find weakly significant linear and quadratic seasonal effects (linear: $P = 0.013$, quadratic: $P = 0.041$) before Bonferroni correction.

Lastly, Lake and Parker used domestic chicken while we used the red junglefowl. Domestic chickens are strongly artificially selected for meat, eggs or sperm. Male domestic fowls may have different sperm investment strategies compared to their ancestors, red junglefowl.

We further analysed that whether this difference is caused by age. The age of year one

males is 3.7 ± 1.6 , while the age of year two males is 4.5 ± 0.9 . The difference of two groups is not significant. From our seasonal results, age is not a significant factor to explain the seasonal pattern. However, from other studies of red junglefowl population, a decline in sperm number was seen when the males aged ⁵⁹. But as the age of two groups of males is similar, we would not expect an age effect. As for the courtship rate, males courted females more in the midday of year one but did not produce ejaculate. In contrast, males rarely courted females in the midday of year two but produced a lot of ejaculates. The courtship rate follows an opposite pattern with the sperm allocation, which suggests males courted more when he was not very interested in mating. In red junglefowl, the overall courtship rate was positively correlated with female choice and mating success ⁶⁸. It is predicted that males should court more for preferred females. The reason might be when the male was very keen to mate, he would not waltz but jumped directly onto my legs and bit female's comb or neck feather. We had to push males away from the females but he would try all means to approach the female. But when the male was interested but not so interested, he would waltz a lot and mated with the female when we presented the female in a soliciting position.

Furthermore, the inconsistency in Lake and our experiments may suggest that circadian patterns of male ejaculate expenditure are plastic. For example, in female red junglefowl, in female-biased groups, sexual harassment was relaxed, and they initiated mating more often in the evening. However, in male-biased groups, females solicited more in the morning and kept a long distance from males in the evenings ⁶⁹. Males could also adjust their circadian patterns according to females. The plasticity in the circadian of sperm allocation is worth further exploration.

Conclusion

In conclusion, patterns of ejaculate investment of male red junglefowl displayed a strong seasonal signature, with sperm numbers peaking in the middle of the breeding season. However, we found no evidence of circadian patterns, which contradicts early

studies in domestic fowl, and possibly reflects differences in experimental approaches. Collectively, our results reveal the importance of considering temporal patterns at different scales when studying the functional significance of male ejaculate investment strategies.

Appendix

Lake and Parker's work were done in 1940s-1950s. Their data analysis was not as advanced as it is now. So I re-do the analysis using their original data.

Lake: sperm number is standardised against the highest value that male produced in three different times of the day to control for inter male variance. Then I did a linear mixed model. The response variable is the standardised sperm number. The fixed effect is the Time of the day. The random effect is Male ID. Males produced significantly more sperm in the afternoon than in the midday (Table 1).

Parker: sperm number are standardised against the highest value that male produced in three different times of the day to control for inter male variance. I did a two-tailed paired-t-test. No significant term was found (Table 1).

Table A1. Standardised sperm number for Lake and Parker's experiments

	Tests	P value	Number of males
Lake's data	Linear mixed model (Standardised sperm ~ Time of the day + (1 Male.ID))	0.016	12
Parker's data	Two-tailed Paired t-test	0.530	6

Post-hoc (Lake): Noon vs Afternoon P=0.026

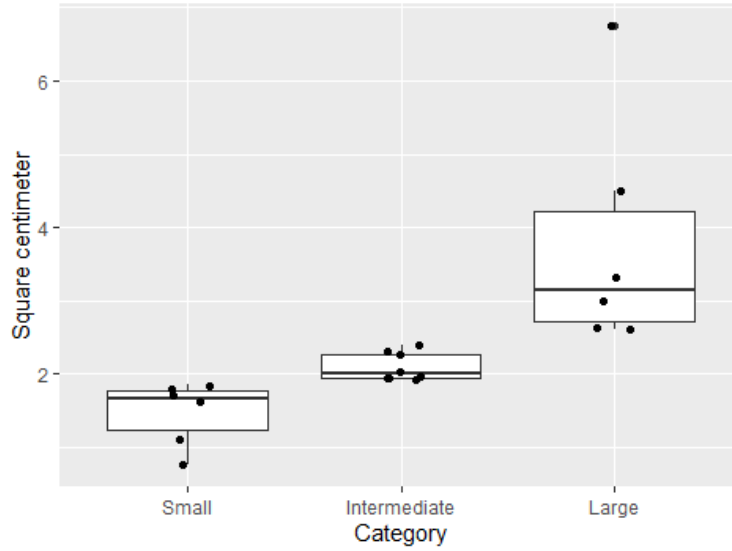


Figure A1. Female comb size: six large-comb (mean $\pm$ s.e.: $3.80 \pm 0.66 \text{ cm}^2$), six small-comb ($1.47 \pm 0.18 \text{ cm}^2$) and eight intermediate-comb ($2.10 \pm 0.07 \text{ cm}^2$) females.

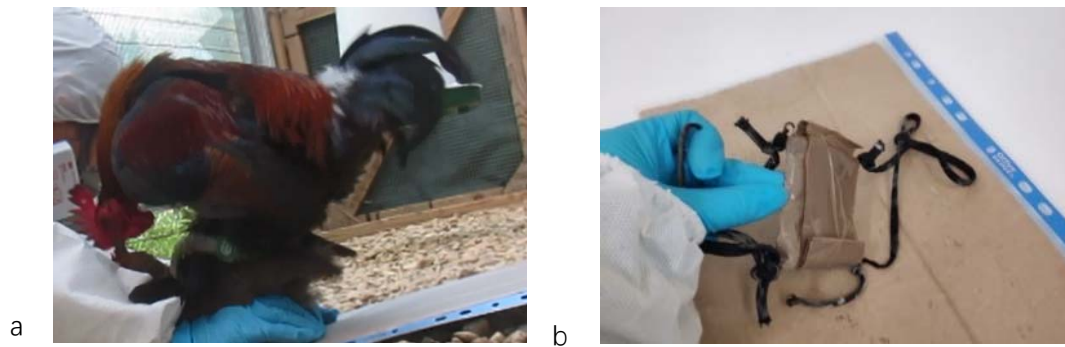


Figure A2. (a)(b) semen collector attached to females used by us.

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Chapter 3. Mechanisms of Male Ejaculate Expenditure in Relation to Male Social Status

Abstract

When females copulate with multiple males (polyandry), sperm competition occurs, and male reproductive success is determined by the ability of a male to mate with females and the ability of his ejaculates to out-compete those of other males over fertilisation. Sperm number and sperm fertilising efficiency are two primary determinants of the outcome of sperm competition. Theory predicts that males gain mating opportunities through traits acting at pre-copulatory levels. Little however is known about the way in which male investment in pre-copulatory competition is related to male expenditure in natural ejaculates. Here, we experimentally tested (a) whether dominant males invested fewer sperm or less motile sperm than subordinate males; (b) whether males who move up the social rank decreased sperm allocation or sperm motility, in male red junglefowl, *Gallus gallus*, where male access to mating opportunities is modulated by male social status and the outcome of sperm competition is determined by both sperm number and sperm swimming velocity. We first established pairs of males, and investigated the number and swimming velocity of sperm allocated by the dominant and subordinate member of each pair to a natural mating. We then re-assembled males across pairs, by assorting males of similar status, to generate four categories of social status (i.e. males that were consistently dominant or subordinate in both pairings, and males that changed status from dominant to subordinate or vice versa), and again investigated sperm numbers and swimming velocity in natural ejaculates. We did not find any difference in sperm number or sperm swimming velocity associated with initial male social status, nor any change in these two parameters following a male pair re-assembling. The results challenge theoretical predictions of direct trade-offs between male investment in pre- versus post-copulatory competition, and suggest that the relationship between social status and ejaculate expenditure may be more complex than originally assumed.

Introduction

Throughout the animal kingdom, females of many species copulate with multiple males (polyandry) so that the sperm of different males have the opportunity of fertilising the same set of ova^{1,2}. Polyandry therefore generates sexual selection on males both before and after copulation^{3,4} and as such plays a fundamental role in the evolution of sexual strategies. Investigating the mechanisms regulating the expression and interactions between traits under pre- (i.e., secondary sexual traits) and postcopulatory (i.e. ejaculates) sexual selection is important to understand the causes of variation in reproductive success within populations and the processes underpinning the evolution of reproductive strategies^{5,6}.

Intrasexual competition for access to sexual partners promotes the evolution of a wide array of traits⁷. Premating traits are selected for the ability to access and choose partners conveying the highest reproductive benefits. In males, where intrasexual competition is often more intense, precopulatory sexual selection has led to the evolution of sexual ornaments to attract females, and weapons or larger body sizes for male-male competition^{3,7}. Polyandry forces the ejaculate of different males to compete for fertilisation (sperm competition). Postcopulatory sexual selection promotes male traits that convey an advantage in sperm competition and help a male increase his share of paternity in the offspring produced by his sexual partner (s)^{8,9}. In many organisms the outcome of sperm competition is strongly influenced by the number of sperm inseminated by rival males and the fertilising efficiency of their sperm^{2,5,10-14}.

In social species, social status results in an unequal distribution of contested resources, such as food, territories, and mates. Here male access to mating opportunity is strongly determined by the social status of a male and his position within the hierarchy. Socially dominant males often sire more offspring in the population^{15,16}. For example, six species of male macaques, *Macaca*, obtain advantageous reproductive output through the acquisition of high social status in their groups¹⁷. In cooperatively breeding wolf,

Canis lupus, reproductive rates are lower for social subordinates than for dominants, and it is common for reproduction in subordinates to be completely suppressed¹⁸. This reproductive advantage can be determined by multiple factors. Dominant males have disproportionately more mating opportunities which enable them to mate with more females and to re-mate with them at a higher rate. Dominant males can also better guard their partners from subordinate males and are more likely to prevent subordinate males from mating. Even in socially monogamous species, *Poecile atricapillus*, dominant males can have more extra-pair copulations¹⁹. Females pairing to low-ranking males constructed nests near the territory edges of neighbouring high-ranking males. Collectively, these effects are likely to reduce the risk (the probability of competing with rival ejaculates) and intensity (the number of rival ejaculates competing with focal ejaculate) of sperm competition faced by the ejaculates of dominant males. Subordinate males on the other hand, are more likely to face sperm competition because of their limited ability to monopolise access to females.

Both pre- and post-copulatory traits are costly. A limited budget should be diverted to either pre- or postcopulatory traits creating a trade-off. Increased male contest competition or sexual attractiveness has been shown to reduce male longevity (i.e. in dung beetle, *Onthophagus binodis*)²⁰. similarly, ejaculates are costly to produce²¹⁻²³ and in numerous species the sperm reserves of males can become depleted, which limits their reproductive success. In addition, nutritional stress has been shown to reduce the lifetime number of mating, testes size, ejaculate quality and competitive fertilisation success^{24,25}. For example, in the Indian meal moth, *Plodia interpunctella*, resource-limited males were smaller, and had decreased the lifetime number of matings²⁶. Dominance is related to the development of weapons and body size, which is presumably energetically costly. Thus dominance may trade-off with ejaculate expenditure. Theory therefore predicts a trade-off between male investment in pre- and post-copulatory competition²⁷. Dominant males, which attain reproductive success largely through their advantages in precopulatory competition and face limited levels

of sperm competition, are predicted to invest less in their ejaculates, while subordinate males which have fewer mating opportunities and face higher levels of sperm competition are predicted to invest more in their ejaculates ²⁸⁻³⁰. The optimisation of these trade-offs has influenced the diversification of sexually selected traits across species and the evolution of alternative mating tactics within species ^{31,32}. For example, *Onthophagus* horned beetles, males that were prevented from developing horns grew larger testes and invested relatively more in testes growth than males who were allowed to grow horns ³¹.

Consistent with this idea, some studies have found a negative relationship between male social status and sperm numbers or sperm fertilising efficiency.

In the European ocellated wrasse, *Symphodus ocellatus*, parental males put considerable effort into obtaining privileged access to mates and into paternal care. This investment is parasitized upon by small- and medium-sized males termed sneakers and satellites, respectively ³³. Although parental males specialise in the behavioural effort, sneakers put more efforts primarily into gonads. In the externally fertilising, chinook salmon, *Oncorhynchus tshawytscha*, loss of social status leads to males increasing sperm swimming velocity within 48 hours following a social challenge, a rapid response mediated by investment in seminal fluid components ³⁴. Similarly, in the Arctic charr, *Salvelinus alpinus*, the male who became dominant in male dyads produced slower sperm four days after the establishment of the dyad ³⁵. In most of these studies however, semen samples were collected through manual manipulations and thus do not necessarily reflect male investment in natural ejaculates.

However, other studies showed social status is not correlated or positively correlated with sperm investment or sperm quality. For example, in the triple fin blenny, *Axoclinus nigricaudus*, no difference in the relative investment into spermatogenesis was detected between dominant (territorial) and subordinate (nonterritorial) satellite males³⁶. There

could also be a positive relationship between dominance and sperm numbers or fertilising efficiency where all males try to optimise the trade-off in a similar way. Dominant males are in better condition and invest more into both pre- and postcopulatory competition. For example, in mice, *Mus musculus*, subordinate males had significantly lower motility sperm than dominant males ³⁷.

In this chapter we conduct an experiment to study the relationship between male social status and patterns of expenditure in natural ejaculates in a population of red junglefowl, *Gallus gallus*. The red junglefowl belongs to the Phasianidae family ³⁸ and is the main ancient ancestor of the domestic chicken, *G. domesticus* ³⁹. The mating system of red junglefowl is polygynandrous, which leads to sperm competition. In natural conditions, males form dominance hierarchies, and social status mediates access to females, placing dominant and subordinate males in favoured and disfavoured mating roles, respectively ⁴⁰. Dominant males have higher copulation success and face a lower risk of sperm competition than subordinate males, but females frequently copulate with multiple males, generating intense postcopulatory sexual selection⁴⁰. When females are polyandrous, the number and velocity of sperm that males inseminate relative to rivals determine fertilisation success ⁴¹. The promiscuous nature of the breeding system and the high frequency with which males can copulate (see Chapters 2, 4 and 5), can lead to sperm depletion. It has been previously shown that males of this species, especially dominant individuals, are economical with their gametes, adjusting the number of sperm they ejaculate according to their social status ⁴². Males who won the agonistic contest twice decreased their sperm velocity ⁴³.

Aims

The aims of this study were to determine the relationship between male social status and two measures of natural ejaculate expenditure: (i) sperm numbers, and (ii) sperm swimming velocity. We aim to answer three questions. Firstly, whether dominant males invest less or lower quality sperm. Secondly, whether males in different social

categories have different sperm number and quality investment strategies. Thirdly, whether status change causes a change in sperm investment or sperm quality.

Methods and materials

We studied a population of red junglefowl at the John Krebs's Field Station (Oxfordshire), which was founded in 2006 from other captive populations. All sampling of birds was conducted in accordance with approved experimental protocols under Home Office licence (PPL P50402706). Males were kept in individual cages for six days with *ad libitum* access to food and water prior the beginning of the experimental trial at all times. We have used the controlled copulation to collect natural ejaculates from male red junglefowl. We used 40 males (all available males that were trained to the controlled copulation) from April to June in 2018 and reused four males in June in 2019. At the onset of a trial, pairs of males were assembled by placing two males together in a new isolated pen. Prior to the experiments, males were kept in single-sex pens of around 12 birds. Males in each group of 4 were intentionally selected from different pens to avoid social familiarity if possible. All males used in the experiments were non-virgins and they were sexually rested for at least 48 hours. Males were not kept visually nor vocally isolated.

To assess the social hierarchy in each male pair, we observed the social interactions of the males for two days, and established their status based on the outcome of agonistic interactions. We observed each pair interact for one hour per day for two days. After the period of observation, males were returned to their isolated pen under the regulation of the project license to avoid injury from the other male. We recorded the number of chasing, pecking, flapping wings, lowering one wing to the ground to make a sound (waltz) and crowing. Males who show more of these behaviours are more dominant ⁴⁴. If the dominance status was not clear by the end of day two, we would add one period of observation on day three (30 pairs only interacted for two days and 14 pairs interacted for three days). Otherwise, on day 3, they were exposed to each other for only 10 minutes to familiarise with each other before collecting ejaculate. Then on day three we collected one ejaculate each male via controlled copulation. Males in each pair had different females. Observation was done from 2-4pm and ejaculate collection from each

male was done from 4-5pm.

There are mainly two non-invasive methods to collect semen from male chicken, abdominal massage, and controlled copulation. Abdominal massage reflects the majority of the extragonadal sperm reserves of a male. Controlled copulation reflects a male's propensity to allocate sperm to a particular female. We measured ejaculate expenditure using a well-established protocol based on the experimental exposure of a male to a female fitted with a harness covering her cloaca ⁴⁵. We randomly chose a female from a group of available healthy females and eleven females were used in this experiment. A female was held for one minute facing the focal male in order to enable the male to inspect her. Then the female was turned around and held in a soliciting position, placed on a plastic substrate and with a harness covering her cloaca to facilitate ejaculate collection without contact with the female reproductive tract. We terminated the trial if the male did not mate for 15 minutes. After each copulation, we collected ejaculates into an Eppendorf tube using a Gilson pipette.

Sperm motility strongly correlates with the fertility¹¹. Average path velocity (VAP) is one of the main parameters to measure sperm motility. Thus, we measured sperm fertilising efficiency as VAP. We measured the VAP within ten minutes after ejaculate collection using computer-assisted sperm analysis (CASA) using a standard protocol. We mixed 180 μ l Dulbecco's Modified Eagle's medium and 20 μ l Standard Foetal Bovine Serum and then put the mixture in 41 °C water bath. We transferred 1 μ l semen into the mixture and fully blended it. Then we transferred 2 μ l mixture to a Leja 10 microscope slide (microptic company) placed on a heated microscope stage set at 41 °C which was connected to a digital video camera set at 10 $\times$ magnification, and measured sperm average path velocity using microptic sperm class analyser: SCA v.6.2.0.15 (Capture 25 images/second; tracking objects between 50 and 200 microns; Optics: Ph-; chamber: Leja 10; Scale: 10 $\times$). A minimum of 200 individual sperm cells will be tracked for each sample across at least three different fields. Sperm sometimes bumped

into each other which may result in a lower VAP. Therefore, in the data analysis, we added the concentration as a fixed effect for VAP to control for the potential effect.

Within roughly three hours of collecting the ejaculate, we diluted 2.5 μ l of semen into 197.5 μ l of Phosphate-buffered saline (PBS) and transferred 70 μ l of the mixture to a spectrophotometer set at 595nm to measure the concentration of sperm within the ejaculate through the light absorbance. We used a standard curve to estimate the sperm concentration and measured ejaculate volume using a Gilson pipette. We computed the raw sperm number using sperm concentration times volume. More details of this protocol can be found in chapter 2.

On day four, we reconstituted male pairs by swapping males across pairs, matching two dominant and two subordinate males together. We observed the social interactions of the males for two days and established their status. Again, if the hierarchy was not clear by the end of day five, we would add one period of observation on day six. This manipulation meant that in each reconstituted male pair, one of the two males would change status, creating four social categories, males that were dominant across both pairs (DD), males that were subordinates in both pairs (SS), males that were dominant in the first pair and subordinate in the second (DS), and males that were subordinate in the first pair and subordinate in the second (SD). Subsequently, on day six, we collected ejaculates again. Males in one pair got two different females (i. e. dominant male a got female A and subordinate male b got female B) to avoid the confounding factor of female mating history since males were not visually isolated. Males in the other pair got the same two females (i. e. dominant male c got female B and subordinate male d got female A). On day six, males got the same female that male had in the first trial to control the female effect. Observation was done from 2-4pm and ejaculate collection from each male was done from 4-5pm. Here is the diagram of the design of the experiment (Fig. 1).

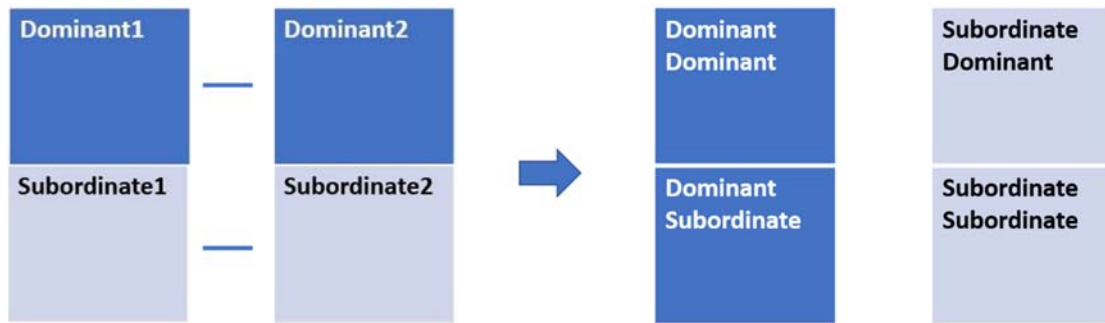


Figure 1. We randomly assigned males into two pairs and observed their status based on the outcome of agonistic interactions. Then we reconstituted male pairs by swapping males across pairs, matching two dominant and two subordinate males together, forcing one male in each pair to change status.

Four males that were used twice in 2018 and 2019 were grouped with males that they never encountered in previous trials. Six males did not ejaculate for both mating trials and were excluded. The age of males included in the dataset is 4.2 ± 1.6 year-old.

Statistical analysis

We analysed data using the statistical software R with the lme4, stats, lsmeans, dplyr, ggplot2, car, packages.

We were trying to answer three questions. Firstly, whether dominant males invest less or lower quality sperm. Secondly, whether males in different social categories have different sperm number and quality investment strategies. Thirdly, whether status change causes a change in sperm investment or sperm quality.

Sperm number

- (i) *Question 1 whether dominant males invest fewer sperm compared to subordinate males*

We standardised sperm number by dividing the sperm number against the highest sperm number that male produced across two trials. Therefore, the standardised sperm number ranges from 0 to 1. To answer the first question, we fitted one generalised linear mixed model with a binomial error structure for standardised sperm. Stage 1 was the first trial and Stage 2 was the second trial. We only selected Stage 1 where they had one agonistic encounter with either dominant or subordinate social status. The response variable is the standardised sperm number. Male social status (Dominant/Subordinate) was entered a fixed effect.

Group represents a block of four males including one DD, one SS, one DS and one SD. Males in each group were tested on the same day. There were eleven groups. Pair represents a pair of two males who had the agonistic encounter. Within each group, there were four different pairs which were labelled as 1 – 4. Pair nested within the group. Group and group pair interaction were entered as random effects to deal with non-independence in the data (when one individual is dominant, another is inevitably subordinate). We used drop1 function (Chisq) to drop out one term each time and tested the significance of each term by comparing the model with and without that term using

anova type II analysis. Chi-square distributions have only one tail and therefore the significance values were one-tailed.

```
glmer(Standardised.sperm~Dominance++(1|Group:pair)+(1|Group),family=binomial)
```

(ii) *Question 2 whether males in different social categories have different sperm investment strategies*

To answer the second question, we fitted one generalised linear mixed model with a binomial error structure for standardised sperm. We only selected Stage 2 where they had two agonistic encounters and were categorised as DD, SS, DS, and SD. The response variable is the standardised number. The fixed effects are the Social category and sperm concentration. The random effects are their group ID and group by pair interaction. We used drop1 function (Chisq) to drop out one term each time and tested the significance of each term by comparing the model with and without that term using anova type II analysis.

```
glmer(Standardised.sperm~Social category+(1|Group:pair)+(1|Group), family = binomial)
```

(iii) *Question 3 whether status change causes a change in sperm investment*

To answer the third question, we fitted one linear mixed model for the change in standardised sperm. The response variable is the change in standardised sperm number (standardised number in 2nd copulation minus standardised sperm numbers in 1st copulation). We entered the Social category as a fixed effect. Group ID was entered as a random effect. To control for the regression to the mean effect, that if, by chance, a male produces a large ejaculate in the first trial, containing more sperm than the male produced on average, the second copulation is likely to represent a reduction. Therefore, we entered the standardised sperm number produced in the first trial to control for this potential effect. We used drop1 function (Chisq) to drop out one term each time and tested the significance of each term by comparing the model with and without that term using anova type II analysis.

$\text{lmer}(\text{difference in Standardised sperm} \sim \text{Sperm1} + \text{Social category} + (1|\text{Group}))$

VAP

- (i) *Question 1 whether dominant males invest sperm of lower VAP compared to subordinate males*

To answer the first question, we fitted one linear mixed model for VAP. We only selected Stage 1 where they had one agonistic encounter with either dominant or subordinate social status. The response variable is VAP. The fixed effects are their social status (Dominant/Subordinate) and sperm concentration to control for potential sperm density effect. The random effects are their group ID and group by pair interaction. We used drop1 function (Chisq) to drop out one term each time and tested the significance of each term by comparing the model with and without that term using anova type II analysis.

$\text{lmer}(\text{VAP} \sim \text{Dominance} + \text{Concentration} + (1|\text{Group:pair}) + (1|\text{Group}))$

- (ii) *Question 2 whether males in different social categories have different ejaculate expenditure strategy in terms of VAP*

To answer the second question, we fitted one linear mixed model for VAP. We only selected Stage 2 where they had a second agonistic encounter and were categorised as DD, SS, DS, and SD. The response variable is VAP. The fixed effects are the Social category and sperm concentration. The random effects are their group ID and group by pair interaction. We used drop1 function (Chisq) to drop out one term each time and tested the significance of each term by comparing the model with and without that term using anova type II analysis.

$\text{lmer}(\text{VAP} \sim \text{Social category} + \text{Concentration} + (1|\text{Group:pair}) + (1|\text{Group}))$

- (iii) *Question 3 whether status change causes a change in ejaculate expenditure strategy in terms of VAP*

To answer the third question, we fitted one linear mixed model for the change in VAP.

The response variable is the change in VAP (Stage 2 minus Stage 1). The fixed effect is Social category. The random effect is their group ID. To control for the regression to the mean effect, we entered the VAP in the first trial to control for the effect. We used drop1 function (Chisq) to drop out one term each time and tested the significance of each term by comparing the model with and without that term using anova type II analysis.

`lmer(difference in VAP~VAP1+Social category+(1|Group))`

Change in status within social categories

Furthermore, to analyse the change in status data within social categories, we used four one-sample two-tailed t-tests to test whether sperm investment change deviates from zero and four one-sample two-tailed t-tests to test whether sperm motility change deviates from zero. The corrected threshold after Bonferroni correction is 1.25%.

Results

For all models, no significant effect of Dominance, Social category or status change was found (Table 1 – 2, Fig. 2 – 3.).

Sperm number

Males who invested lots of sperm in the first stage reduced sperm investment in the second stage and males who invested fewer sperm in the first stage increased sperm investment in the second stage. This effect is significant (Table 1c).

Table 1. The analysis of variance (ANOVA) table

(a) glmer on the standardised sperm number and social status of the Stage 1

Response variable	Factors	df	Chisq	P value
Standardised sperm	Dominance	1	0.041	0.840

(b) glmer on the standardised sperm number in different social categories of the

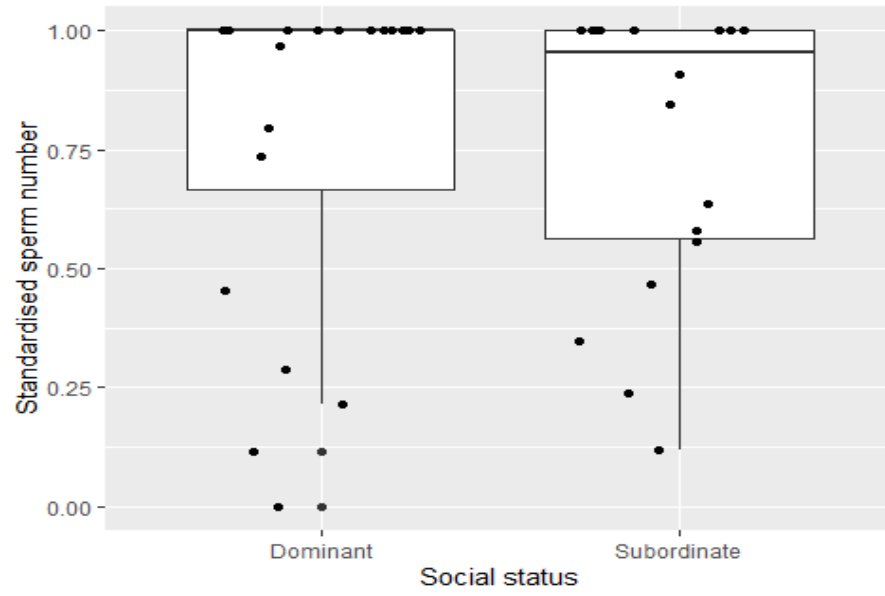
Stage 2

Response variable	Factors	df	Chisq	P value
Standardised sperm	Social category	3	0.777	0.855

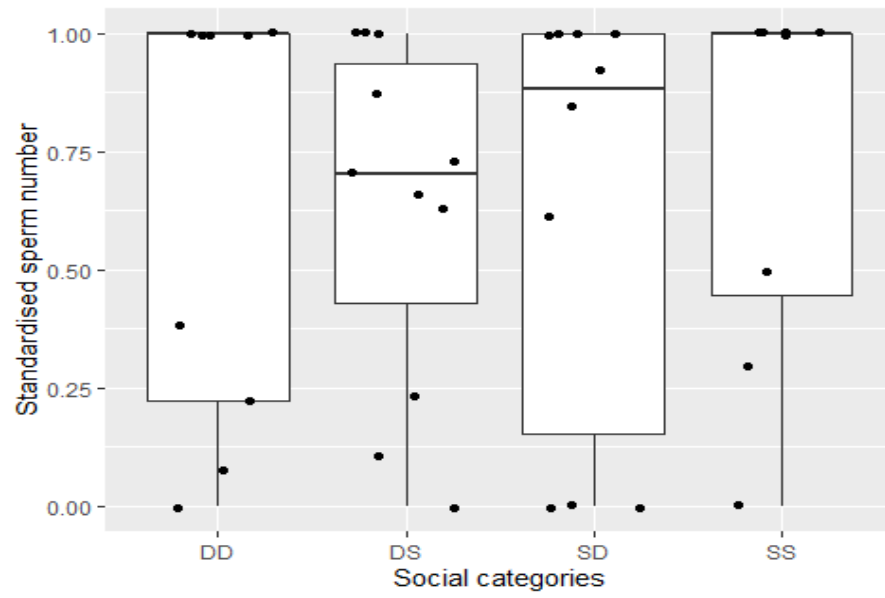
(c). lmer on the standardised sperm change in different Social categories

Response variable	Factors	df	Chisq	P value
Difference in the Social category		3	1.801	0.615
standardised sperm	Sperm in Stage 1	1	55.284	<0.001
number				

a



b



c

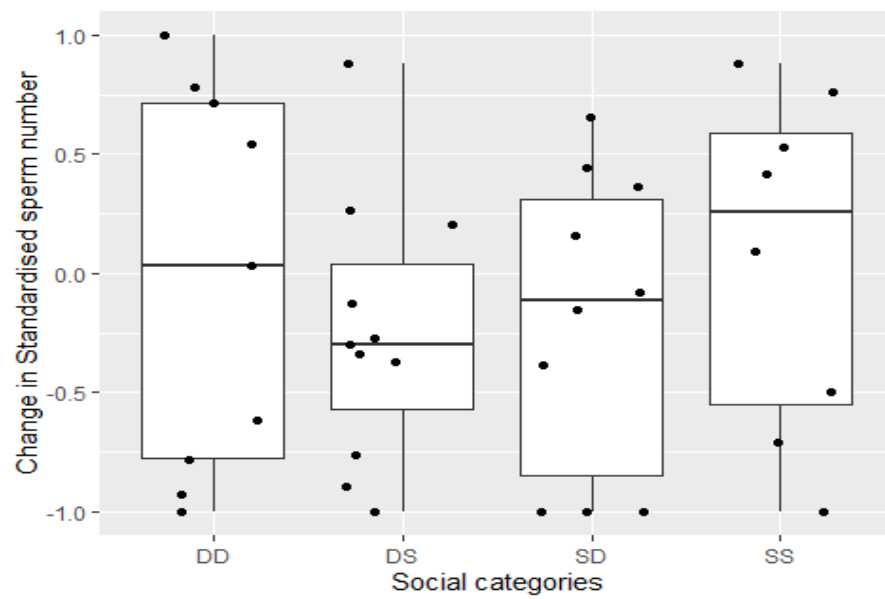


Figure 2. (a) This is a boxplot of the standardised sperm number of the males that became dominant and subdominant in the first social encounter (Stage 1). We standardised sperm number by dividing the sperm number against the highest sperm number that male produced in two stages. Dominant males tended to invest more sperm but this difference was not significant.

(b) This is a boxplot of the standardised sperm number of the males in each Social category (DD, DS, SD, SS) in stage 2. Four social categories did not show a significant difference.

(c) This is a boxplot of the change (stage 2 – stage 1) of the standardised sperm number of the males in each Social category (DD, DS, SD, SS). DS and SD males decreased sperm investment, SS males increased sperm investment while DD males remained the same. But none of the differences was significant.

It shows the “minimum”, first quartile (Q1), median, third quartile (Q3), and “maximum”. On top of the boxplot, there are jittered points. Each male contributes one point.

VAP

Males increased their VAP if they changed from subordinate to dominant, decreased their VAP if they changed from dominant to subordinate (Fig. 3c). However, this effect was not significant (Table 2c). Sperm concentration did not influence VAP (Table 2a-b). Males invested higher VAP sperm in the first stage reduced sperm investment or VAP in the second stage and males invested lower VAP sperm in the first stage increased VAP in the second stage. This effect is significant (Table 2c).

Table 2. The analysis of variance (ANOVA) table

(a). lmer on the VAP and social status of the Stage 1

Response variable	Factors	df	Chisq	P value
VAP	Dominance	1	0.126	0.723
	Concentration	1	1.478	0.224

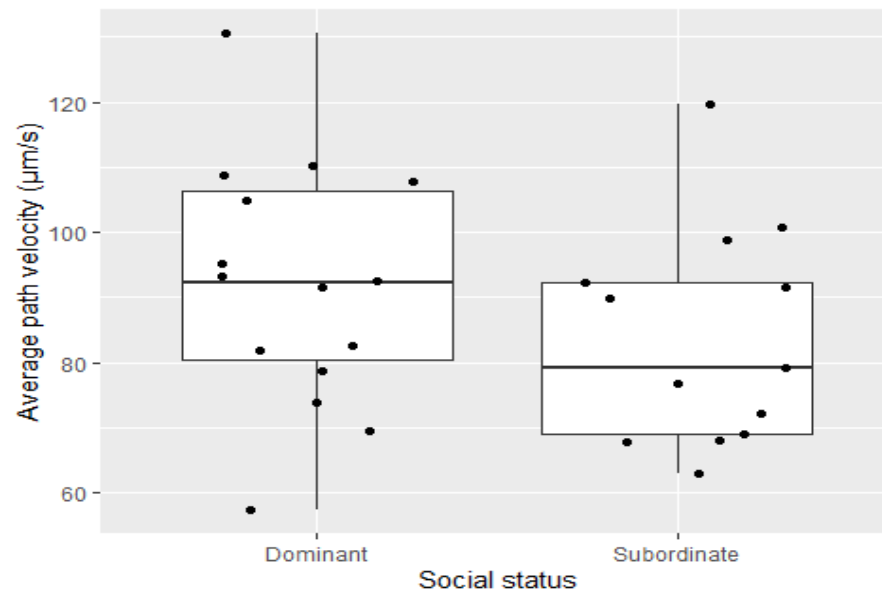
(b) lmer on the VAP in different social categories of Stage 2

Response variable	Factors	df	Chisq	P value
VAP	Social category	3	0.866	0.834
	Concentration	1	0.058	0.810

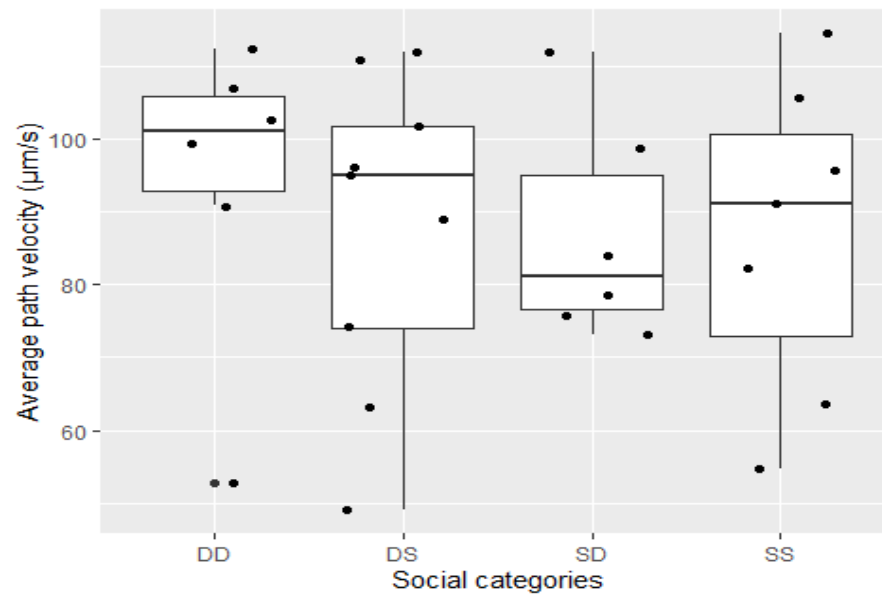
(c). lmer on the VAP change in different social categories

Response variable	Factors	df	Chisq	P value
Difference in VAP	Social category	3	0.867	0.833
	VAP in Stage 1	1	6.489	0.011

a



b



c

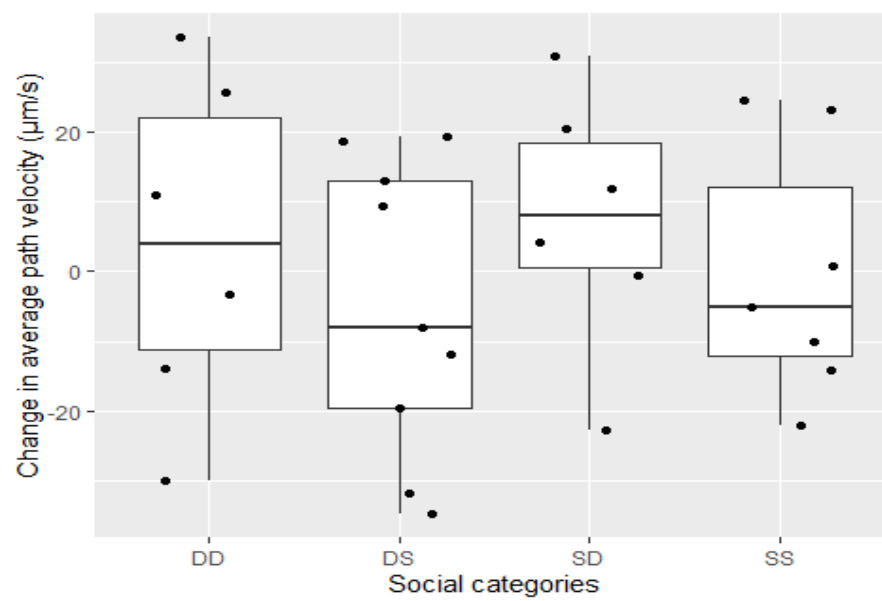


Figure 3. (a) This is a boxplot of the average path velocity of the males that became dominant and subdominant in the first social encounter (Stage 1). Two social categories did not show a significant difference.

(b) This is a boxplot of the average path velocity of the males in each Social category (DD, DS, SD, SS) Stage 2. Four social categories did not show a significant difference.

(c) This is a boxplot of the change (stage 2 – stage 1) of the average path velocity of the males in each Social category (DD, DS, SD, SS). DS and SS males decreased sperm investment, SD males increased sperm investment while DD males remained the same. But none of the differences was significant.

It shows the “minimum”, first quartile (Q1), median, third quartile (Q3), and “maximum”. On top of the boxplot, there are jittered points. Each male contributes one point.

Change in status within social categories

Within each social categories, we did not find a significant effect in terms of the standardised sperm number or VAP.

Table 3. one-sample t-tests

Response variable	Social categories	df	t-statistic	P value
Standardised sperm	DD	8	-0.102	0.921
	SS	10	-1.483	0.169
	DS	9	-1.002	0.343
	SD	7	0.238	0.819
VAP	DD	5	0.387	0.714
	SS	8	-0.730	0.486

DS	5	0.962	0.380
SD	6	-0.062	0.952

Discussion

The aims of this study were to determine the relationship between male social status and sperm numbers/sperm swimming velocity. Our hypotheses are firstly, whether dominant males invest less or lower quality sperm than subordinate males; secondly, whether males in DD social categories invest least or lowest quality sperm while males in SS social categories invest most or highest quality sperm and SD, DS males invest sperm in between. Thirdly, whether a drop in social rank causes an increase in sperm investment or sperm quality while a rise in social rank causes a decrease.

However, no significant effect was found for either sperm number or VAP. Males that changed their status from subordinate to dominant increased their sperm VAP, while males who changed their status from dominant to subordinate decreased their sperm VAP, though the differences were not significant. These results were not consistent with theoretical expectations and the findings of some previous work which have shown that dominant males tend to produce less motile sperm than subordinate⁴³. Multiple reasons may explain the findings of this study.

Firstly, dominant males tend to have better access to food and water. Therefore, instead of trade-offs, subordinate males may be constrained to generate high-quality sperm. For example, for a group-living reef fish, *Paragobiodon xanthosomus*, competition over food and conflict over social rank are responsible for the regulation of subordinate growth. Subordinates were forced to reduce their own food intake, and growth⁴⁶. Reduction in food intake may have a negative effect on semen. Semen volume of boars, *Sus scrofa domesticus*, was sharply reduced when feed intake was severely restricted. Ejaculate components decrease during ageing and the decrease was significantly faster in limited-fed males⁴⁷. If this is the case, the correlation between social status and ejaculate production is positive. However, in this experiment, males were kept together for only one hour per day. They can feed *ad libitum*. Food and water were not a problem.

Differential access to food and water is therefore unlikely to have confounded these findings.

Secondly, subordinate males are chronically stressed and may suffer from high immunity costs. These physiological costs may prevent subordinate males from producing high-quality ejaculate. After agonistic encounters, the subordinate males shed more blood over the comb, less energy may be diverted to reproduction. Though theory predicts that subordinate males may have upregulated ejaculate, at the same time, immunity requires more energy. If less energy is diverted to reproduction, sperm production is expected to drop. Therefore, the effects on ejaculate production may cancel each other, resulting in no significant effects. Subordinate male rats, *Rattus norvegicus*, showed symptoms of depression and their life-spans are shortened. Basal corticosterone levels were sharply higher in subordinates compared to dominant males⁴⁸. Exposure of rainbow trout, *Oncorhynchus mykiss*, to repeated stress during reproductive development resulted in a significantly lower sperm counts and lower quality of sperm⁴⁹. In red junglefowl, dominant males had better immune response than subordinate males⁵⁰. Subordinate male red junglefowls might be stressed by the presence of dominant males. Chronic stress hypothesis may be able to explain why males who changed their status from subordinate to dominant increased their sperm VAP, while males who changed their status from dominant to subordinate decreased their sperm VAP.

Thirdly, ejaculate and social status may be two independently evolved traits. Thus, manipulating social status would not change the sperm number or sperm motility.

Here I presented a summary table comparing the two most relevant studies with our study and discussed the key differences. Pizzari et al.'s study showed that the sperm quality dropped in males that were dominant both before and after the social challenge

while that of males that were subordinate remained constant. Cornwallis et al. found that the velocity of sperm declined faster over successive copulations in dominant males than in subordinate males⁵.

	Our experiment	Pizzari's experiment ⁴³	Cornwallis's experiment ⁵
Method	Controlled copulation	Abdominal massage	Controlled copulation
Number of ejaculates	1	1	Multiple
Species	Red junglefowl, <i>Gallus gallus</i>	Domestic fowl, <i>Gallus gallus domesticus</i>	Red junglefowl, <i>Gallus gallus</i>
Days kept together	1 hour/day for 3 days	3 days	14 days
Sample size	38	60	11
Results	No effect	Sperm quality dropped in males that won both contests while the sperm quality of males that lost both contests remained constant.	When males were dominant, the velocity of their sperm over successive copulations declined to a greater extent than when they were subordinate.

Firstly, the study from Pizzari et al. used semen collected by massage. The two methods (the abdominal massage and the controlled copulation) capture different aspects of sperm competition. Abdominal massage delivers most of the male extra-gonadal sperm reservoirs out of male's control and results in sperm depletion ^{43,51}. However, males can ejaculate as many as six times in one hour. Therefore, each ejaculate is limited and plastic. The average quality of all sperm might change according to the social status

change, but the quality and quantity of the first ejaculate might not change accordingly. Similarly, another study allowed the male to mate *ad libitum* with the female⁵. The measurement of sperm motility of the pool of a series of ejaculates may be different from that of the first ejaculate. Cornwallis et al. used controlled copulation rather than abdominal massage.

Secondly, Pizzari et al. 's work was done on domestic chicken. Red junglefowl is the wild ancestor of domestic chicken. Behavioural study results differ from species to species, population to population. It is hard to draw a single conclusion. The domestic chicken is a captive species while red junglefowl is a wild species (though kept in captivity in my experiment). The dyad fighting may not resemble the hierarchy determination mechanisms of that in the wild which is more complex. In wild populations, age (but see in male red junglefowl ⁵²), social experience (see in female red junglefowl ⁵³), social connection(see in primates⁵⁴), and etc. may contribute to the establishment of hierarchy. Cornwallis et al. experimented on the red junglefowl.

Thirdly, due to ethical constraints, males were only exposed to each other for no more than 1 hour per day (early termination if a fierce fight was observed or if the hierarchy was clear, they were exposed to each other only 10 minutes before the controlled copulation). One hour might not be enough to trigger a plastic response. In Pizzari et al.'s study, males were kept together for 3 days ⁴³. In Cornwallis et al.'s study, males were kept together for 14 days. While males were kept alone, they tended to act as dominant. In *Drosophila melanogaster*, the presence of rival males prior to mating influenced male's mating duration. The longer the males were exposed to rivals prior to mating, the longer their subsequent mating duration⁵⁵. Hence, insufficient exposure to the competing male might reduce the magnitude of sperm change.

Fourthly, the sample size is not very large, with only 38 males. The small sample size

resulted from the fact that only a limited set of males were used to the controlled copulation, so we were not able to include more males. Pizzari et al. used 60 males and Cornwallis et al. used 11 males. Furthermore, the sampling process might be biased, because only a subset of males was used to the controlled copulation. They might be bolder, more sexually active, or more dominant. All those factors confounded the results.

But we did find the sperm number or VAP in the first trial can influence the change in sperm number or VAP. The higher sperm number or VAP in the first trial, the greater the reduction of sperm number or VAP in the second trial. There are two hypotheses. The first one is the regression toward the mean phenomenon, that statistically if a random variable is extreme on its first measurement, the second measurement tends to get closer to the mean or average ⁵⁶. Therefore if their first measurement is very high, it is unlikely for the second measurement to be as high as the first measurement. Then the sperm number or VAP change tends to be negative. On the contrary, if the first measurement is very low, the second measurement is unlikely to be as extreme as the first measurement, so the change in sperm number or VAP is positive.

The second hypothesis is there is a trade-off of sperm number and VAP between current and future mating opportunities. Because males have a finite amount of sperm, if he used a lot in the first stage, he would have fewer left for the second stage. However, previous studies have shown that it takes a male fowl 48h to completely restore its sperm reservoirs after sperm is completely depleted by manual massage ⁵¹ or by a series of consecutive natural copulations ⁴². In this experiment, the second ejaculate was collected 72 hours after the initial ejaculate. Males' sperm reservoir should have been replenished. Current-future sperm investment trade-offs only happen when the second ejaculate is produced before the sperm reservoir is replenished.

Conclusion

Firstly, we did not find the correlation between male social status and sperm investment/sperm motility. Secondly, we did not find the correlation between male social categories and sperm investment/sperm motility. Thirdly, we did not find the correlation between male social status change and sperm investment/sperm motility. Fourthly, males who invested more sperm or higher-motility sperm in Stage 1 subsequently invested less or lower-quality sperm in Stage 2. These results collectively suggest that the trade-offs between pre-mating and post-mating traits might be more complex than we thought before.

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Chapter 4: Cryptic Male Choice for Ornamented Females

Abstract

When ejaculate investment is costly such as in polyandrous systems characterised by sperm competition, males are expected to invest strategically in mating opportunities based on the reproductive quality of a female. While evidence for such ‘cryptic’ forms of male preferences has been accumulating over recent years, little is known about the mechanisms underpinning male decision-making. In the red junglefowl, *Gallus gallus*, the expression of a fleshy ornament, the comb, signals female fecundity and reproductive investment¹. Accordingly, several studies of red junglefowl and domestic fowl, *G. domesticus*, have shown that when presented simultaneously with females of different comb sizes, socially dominant males preferentially invest in larger-combed females^{2 3}, but little is known about male responses in the more realistic scenario where females are presented sequentially. We designed experiments to test how a male’s previous sexual experience influences his propensity to invest in a new female. In the training phase, males were presented with one large-combed female in each of four consecutive trials in one treatment, and with one small-combed female in each of four trials in the other treatment. In the final trial, males from both treatments were exposed to an intermediate-combed female. Throughout the training phase, males exposed to large-combed females invested significantly more sperm than males exposed to small-combed female, confirming previous findings. Importantly, males from both treatments did not adjust sperm allocation when exposed to the intermediate-combed female in the final trial. Thus, we found a strong carry-over effect whereby intermediate-combed females received more sperm from males that had been previously exposed to large-combed females in their training treatment than from males that had been trained on small-combed females. The functional significance of this response is unclear. A carry-over effect might be an adaptive way to cope with a stable environment or might be a maladaptive consequence of cognitive constraints. These results demonstrate the

importance of previous experience in cryptic male preferences.

Introduction

The Darwin-Bateman paradigm proposes that females are the choosy sex ⁴⁻⁶. However, recent studies have shown that sperm is not always cheap or limitless. While producing an individual sperm cell is much cheaper than producing an individual egg, ejaculates usually comprise millions of sperm and a considerable amount of non-sperm components such as seminal fluid proteins ⁷. Ejaculates may be depleted after multiple matings ⁸, potentially limiting the reproductive success of a male. Males with access to multiple mating opportunities are therefore expected to ameliorate this constraint through economical strategies of ejaculate investment ⁹. Strategies of differential ejaculate investment are particularly important in species with high levels of sperm competition, which requires increased male ejaculate investment ^{7,10}.

Male mating behaviour exhibits great plasticity and can change rapidly in response to changes in the social environment. There is increasing evidence that plasticity enables males to strategically adjust ejaculate investment. For example, in the fruit fly *Drosophila pachea* males compromise the number of sperm in each ejaculate in order to achieve multiple successive matings ¹¹. Squid, *Idiosepius paradoxus*, also have the capacity to modulate the amount of spermatangia delivered in different matings ¹². Therefore, resolving the mechanisms underpinning ejaculate investment decisions under different social contexts is important to further our understanding of sperm competition, sexual selection and sexual conflict.

A key factor expected to drive male ejaculate investment strategies is the reproductive quality of female partners, and males can respond to variation in female quality through different mechanisms of mate choice ^{7,10 13}. Male mate choice can happen both before and after copulation. If it happens before copulation, that is a direct mate choice for mating which is also called premating mate choice. If it happens during/after copulation, that is a mate choice for males to invest sperm and for females to use the sperm

strategically which is called cryptic mate choice, also known as the postmating mate choice. Preferentially investing in higher quality partners represents a cryptic form of male mate choice. A prerequisite for male choice is variance among females ¹⁴. For a long time, it was assumed that there is a considerable variance among males, which serves as the raw materials of sexual selection. The female side is often neglected. But actually females can vary considerably in the quality of their reproductive output, both in terms of the number of eggs and nutrients allocated to each egg ¹⁵. Females possess honest signals to reflect their quality, and males are sensitive to the variance in female quality. For example, female body size in some species correlates with fecundity. Thus, males prefer large or heavy females (i.e., in amphibian ¹⁶, bird ¹⁷, fish ¹⁷ and insects ¹⁸). Firstly, male choice can be influenced by female size. There is a significant positive relationship between female body length and brood size in the Trinidadian guppy, *Poecilia reticulata*. Males tended to mate with the larger female when two females are presented simultaneously ¹⁹. Similarly, male *Drosophila melanogaster* allocated more seminal fluid to larger and more fecund females ³. Secondly, males prefer to mate with more colourful females ²⁰⁻²³ and can adjust their mating decision based on the sexual ornamentations of females. For example, male rock sparrow, *Petronia petronia*, the effort males put into defending the nest was significantly related to female breast patch size, which suggests males can tell the difference of female quality based on their sexual ornamentations and they can adjust the parental investment decisions ²⁴. When being exposed to two females simultaneously, males sparrow were more likely to copulate with and allocate more sperm to the larger sexually ornamented female ⁹. Similarly, female two-spotted gobies, *Gobiusculus flavescens*, have bright yellow-orange bellies during the breeding season, which is not present in males. A study has found that males preferred to mate with more colourful females by showing more courtship displays or spend more time with them ²⁵. In some species, males prefer young females over old ones. Younger females may have better fecundity, less sperm competition risk and less sexually transmitted infections²⁰. Virgin females are usually younger than non-virgin

females. Great snipe, *Gallinago media*, males preferred to mate with virgin females when given the opportunity ²⁶. Males that were exposed to immature females on the other hand, produced ejaculates of low sperm viability ²⁷.

Male preference is modulated by female variation. For instance, male bushcrickets, *Kawanaphila nartee*, are less choosy and less likely to reject female's solicitation when the female variance of a group of available females is low than when that is high ²⁸. Indeed, males of various taxa adjust ejaculates to female quality ^{3 19 27 29 30}. Male mate choice is more likely to operate in a female-biased operational sex ratio (OSR) group where mating opportunities are more abundant. For example, male bushcrickets, *K. nartee*, are faster to mate in a male-biased group than a female-biased group ²⁸.

While the majority of studies investigating male choice uses a simultaneous paradigm to present females, in nature males are more likely to encounter females sequentially rather than simultaneously in nature ^{31,32}. Under sequential encounters, a male must decide how much to invest in the current female. Information required by decision-making might be built up over time ³³. Therefore, males may make their decisions on sperm allocation based on past experience. Ejaculate expenditure in the current female will be influenced by the expected payoff of encountering a new female in the near future. For example, models predict that the preference may be reversed if the mating is delayed ³⁴ due to the possibility of losing an acceptable mate to competitors. Search costs, including energy and time costs, predation risks, can also affect choosiness. For example, in a dusky salamander, *Desmognathus santeetlah*, female fecundity is positively correlated with body size. Males mated with small and large females at similar rates when females were presented sequentially, but prefer to mate with larger females when presented simultaneously ³⁵. In sequential encounters, males need to rely on experience more because the female quality in the past can inform the female quality in the future, while simultaneous encounters are a one-off process.

Animals can rely on the recent experience of exploring environments and estimating the quality of food patch or mate patch to make a decision³⁶. Successive contrast effects illustrate that behaviour is dependent on whether conditions are currently better or worse than they were before. The contrast effects may result from an adaptive response to uncertainty in a changing, unpredictable environment³⁷. In the three-spined sticklebacks, *Gasterosteus aculeatus*, females choose mates based on comparison with previous males³⁸. They preferred males that were better than before. Secondly, past sexual experience may influence current decision-making by mediating homeostatic responses (i.e. hormone levels). Prior experience may associate with homeostatic change which subsequently affects decision making. For example, neural activation may reflect the homeostatic changes associated with different risk experiences. Individual participants experienced low-risk task significantly increased the urge for taking a risk subsequently³⁹. The underlying cognitive mechanisms through which a male's past experience influences current sperm investment remain poorly understood.

In this chapter, we explored the mechanisms modulating male ejaculate investment in sequentially presented females of varying reproductive quality in a population of red junglefowl, *Gallus gallus*. The red junglefowl is the main contributor of the domestic chicken⁴⁰. The mating system of natural populations is polygynandrous, which leads to sperm competition. Female reproductive quality covaries with the size of the female comb, a fleshy ornament. Furthermore, female comb size decreased during incubation which is the sexually unreceptive period¹. Genetic analysis of junglefowl domestic chicken reveals that increased female somatic investment in the comb is associated with an increased reproductive investment, in terms of higher fecundity and larger eggs⁴¹. This could be explained by the fact that female comb mass is associated with female social status and the power over resources in fowl populations⁴². Females with large combs may therefore produce both, more offspring and more successful offspring, and

males preferentially investing in large-combed females may therefore sire more offspring and higher quality offspring⁴¹. Several previous studies have shown that males, particularly socially dominant males, from both red junglefowl and feral domestic populations prefer to mate with-, and allocate more sperm to females with a larger comb, when a large- and small- combed females were presented simultaneously^{30,43,44}.

Aims

The aims of this chapter were twofold. First, we investigated whether males invest preferentially in larger-combed females when sequentially exposed to different females. Second, we tested how the quality of females previously encountered by a male affects his investment in a new female. Our hypotheses were:

1. Under sequential encounters, males will invest more sperm in large-combed females, than in small-combed females.
2. Males that experience a succession of large-combed females will reduce their sperm allocation when exposed to an intermediate-combed female, while males exposed to small-combed females will increase their sperm allocation in an intermediate-combed female.
3. Thus, intermediate-combed females may potentially obtain more sperm from males trained on small-combed females, than from those trained on large-combed females.

Method

We studied a population of red junglefowl at the John Krebs's field station in Wytham, Oxford from June to August 2018. The work was conducted under the regulation of a Home Office project license (PIL: P50402706).

The experiment was conducted from June to July 2018. Prior to the experiments, males were kept in single-sex pens of around 12 birds. All males used in the experiments were non-virgins and they were sexually rested for at least 48 hours. During the experiment, males were kept in single pens but were not visually isolated nor vocally isolated. For the second and third experiment, males were not visually isolated. We randomly assigned 20 sexually mature males to two treatments. In the 'large' treatment, each of 10 males was individually exposed to a single large-combed female in each trial, while in the 'small' treatment the remaining 10 males are exposed to a small-combed female in each trial. In both treatments, each male was exposed to 4 trials, one every other day to allow for replenishment of extragonadal sperm reserves⁴⁵. In each trial a male could mate up to 6 times with the female within a period of 60 minutes. A male was exposed to a different female in each of the 4 trials. After the four training trials, males from both treatments underwent one final trial where each was individually exposed to the same intermediate-combed female and allowed to mate up to 6 times within 60 minutes. In between trials, males were housed in individual pens. The age of males in treatment large is 3.8 ± 0.49 years old and that for treatment small is 4.1 ± 0.53 years old. The difference is not significant. One male in the large treatment did not ejaculate throughout the 5 trials and was excluded. Here is the experimental diagram (Fig. 1).

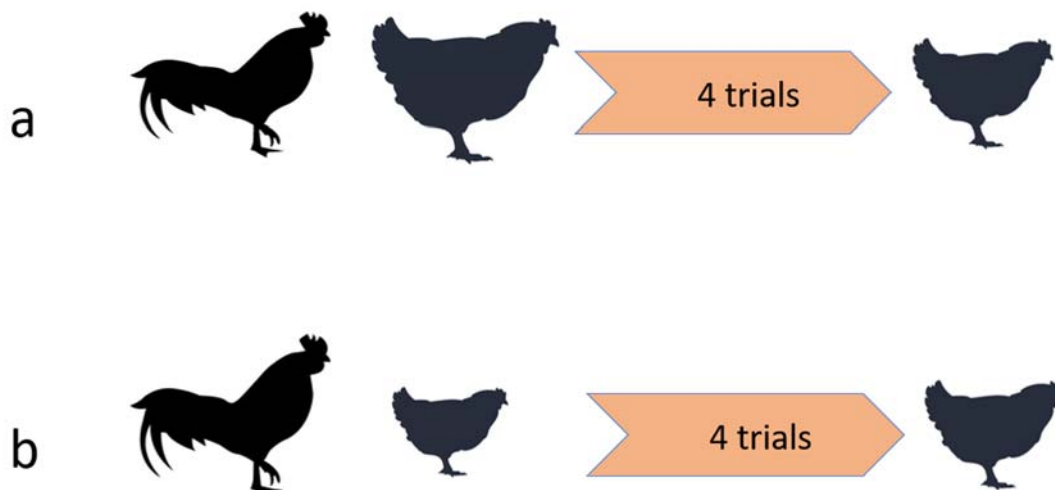


Figure 1. (a) T1 – trained on large-combed females, final trial with intermediate female. (b) T2 – trained on small-combed females, final trial with intermediate female.

During a trial, a female was held for one minute facing the focal male in order to enable the male to inspect her. Then the female was turned around and held in a soliciting position, placed on a plastic substrate and with a harness covering her cloaca to facilitate ejaculate collection by preventing insemination in the female reproductive tract. We recorded the time to ejaculate, which is the time from holding the female in a soliciting position to the first successful ejaculation. We terminated the trial if the male did not mate for 15 minutes. After each copulation, we collected the ejaculate into an Eppendorf tube using a Gilson pipette. Within roughly three hours of collecting the ejaculate, we diluted 2.5 μ l of semen into 197.5 μ l of Phosphate-buffered saline (PBS) and transferred 70 μ l of the mixture to a spectrophotometer set at 595nm to measure the concentration of sperm within the ejaculate through the light absorbance. We used a standard curve to estimate the concentration of sperm within each ejaculate. We measured ejaculate volume using a Gilson pipette. We computed the raw sperm number using sperm concentration times volume. More details of this protocol can be found in chapter 2.

We measured female comb size by taking photos of females' comb with a ruler. Then we measured the area of the comb using image J (v1.51 w), adjusting the scale against

the ruler. We randomly chose four large-combed females (Appendix Fig. A1. mean $\pm$ s.e.: $4.39 \pm 0.085 \text{ cm}^2$), four intermediate-combed females ($2.21 \pm 0.08 \text{ cm}^2$) and four small-combed females ($1.36 \pm 0.26 \text{ cm}^2$. The differences are significant). We used progressively larger combed-females over the four trials of the large treatment, terminating with the two largest combed females in trials 3 and 4. Similarly, we used progressively smaller combed females over the four trials of the small treatment, terminating with the two smallest comb size females in trials 3 and 4. This amplified the difference in comb size between trial 4 and trial 5 where the male was presented with an intermediate-combed female. Within each treatment, variation in female comb size across trials was not a significant predictor of male ejaculate size (Appendix. Fig. A2).

We also attempted to conduct a second experiment to disentangle the role of female quality and female order. This experiment was conducted from July to August 2018, and comprised 16 males across two treatments. Unfortunately however, several males produced few or no ejaculates throughout these trials. This likely reflects the fact that this period coincides with the end of the breeding season. We therefore could not include the data of this second experiment in this chapter.

Statistical analyses

We conducted the analyses using the statistical software R with the lme4, stats, lsmeans, ggplot2, dplyr, car packages.

Training trials

The training phase comprises trials 1-4. We investigated the ejaculate expenditure strategies of males in the first 4 trials of the large and small treatments. Firstly, we standardised the cumulative sperm number that a male produced in a trial by dividing against the largest cumulative sperm number in a single trial across the whole dataset. Then the standardised cumulative sperm number ranges from 0 to 1. We ran a model to test whether the standardised sperm number differs across treatments in the training phase. To do this, we fitted a linear mixed effect model to the data in the training phase with a gaussian error structure, with standardised sperm number with zeros in a trial as the response variable, treatment as a fixed effect, and male ID, Trial, as random effects. Then, we used drop1 function to drop out one term each time and ran an anova chisq-test between the model with and without that term. Chi-square distributions have only one tail and therefore the significance values were one-tailed.

```
lmer(standardised sperm number~Treatment+(1|Trial)+(1|Male.ID))
```

We considered two measures of male choice. The first is for a male to decide whether to mate with the female or not. The second is if the males decide to mate, how many sperm he should allocate to the female, which represents a cryptic form of male choice. Therefore we ran a model to test whether the probability of ejaculation differs across treatments in the training phase. To do this, we fitted a generalised linear mixed effect model to the data with a binomial error structure, with the successful ejaculation as the response variable (a successful ejaculate as “1”, no ejaculate as “0”, six data points in a trial of each male), treatment as a fixed effect, and male ID, Trial, as random effects. Then, We used drop1 function to drop out one term each time and ran an anova chisq-

test between the model with and without that term.

```
glmer(Ejaculate or not~Treatment+(1|Male.ID)+(1|Trial), family=binomial)
```

We ran a model to test whether the probability of mating differs across treatments in the training phase. We fitted a generalised linear mixed effect model to the data with a binomial error structure, with the successful copulation as the response variable (a successful copulation as “1”, no copulation as “0”), treatment as a fixed effect, and male ID, Trial, as random effects. We then used the drop1 function to drop out one term each time and ran an anova chisq-test between the model with and without that term.

```
glmer(Mating or not~Treatment+(1|Male.ID)+(1|Trial), family=binomial)
```

We ran a model to test whether the time to ejaculate of the first copulation differs across treatments in the training phase. To do this, we fitted a linear mixed model to the data in the training phase, with log(time to ejaculate) as the response variable, treatment as a fixed effect, and male ID, Trial, as random effects. Then, we used drop1 function to drop out one term each time and ran an anova chisq-test between the model with and without that term.

```
lmer(log(Time)~Treatment+(1|Trial)+(1|Male.ID))
```

The final trial

We ran a model to test whether the standardised sperm number differs across treatments in the final phase. The final phase only comprises of trial 5. Each male contributes one data point. We fitted a linear model to the data in the final phase with a gaussian error structure, with standardised sperm number with zeros in a trial as the response variable, treatment as a fixed effect. Then we ran an anova F-test of the model. F distributions have only one tail and therefore the significance values were one-tailed. The model was compared to a null model.

```
lm(Standardised sperm~Treatment)
```

We ran a model to test whether the probability of ejaculation differs across treatments in the final phase. To do this, we fitted the a generalised linear mixed model for the final trial, with a binomial error structure, with the successful ejaculation as the response variable, treatment as a fixed effect, and male ID as a random effect. Each male contributes six data points. Then, we used drop1 function to drop out one term each time and ran an anova chisq-test between the model with and without that term.

```
glmer(Ejaculate or not~Treatment+(1|Male.ID), family=binomial)
```

We ran a model to test whether the probability of mating differs across treatments in the final phase. We fitted another generalised linear mixed model for the final trial, with a binomial error structure, with the successful mating as the response variable, treatment as a fixed effect, and male ID as a random effect. Each male contributes six data points. Then, We used drop1 function to drop out one term each time and ran an anova chisq-test between the model with and without that term.

```
glmer(Mating or not~Treatment+(1|Male.ID), family=binomial)
```

We ran a model to test whether the time to ejaculate of the first copulation differs across treatments in the final phase. We fitted a linear model to the data in the final phase with a gaussian error structure, with log(time to ejaculate) as the response variable, treatment as a fixed effect. Then we ran an anova F-test of the model. The model was compared to a null model.

```
lm(log(Time)~Treatment)
```

Predicted value versus observed response

We tested whether male sperm allocation in the final 5th trial could be predicted by male sperm allocation in the training trials 1-4. We used two approaches to compare a male's sperm allocation predicted for trial 5, based on his sperm allocation in the four training

trials with the actual value of sperm allocation observed in trial 5. Firstly, we fitted a linear model of the four training trials in each treatment. We then used the linear model to predict the value of sperm allocation for a 5th trial. We generated a predicted value for each male. If the predicted value was negative, we entered it as zero because negative sperm number does not make sense. Then, we fitted a linear mixed model. The response variable is the standardised sperm number. The fixed effect is Treatment * Phase. Phase 1 is predicted value and phase 2 is the observed value. Male ID was entered as random effect. We used drop1 function to drop out one term each time, and ran anova Chisq-test between the model with and without that term.

```
lmer(Standardised sperm~Treatment*Phase+(1|Male.ID))
```

To explore these results further, we used a second approach. We computed the mean of the standardised sperm numbers across the training trials 1-4, for each male in each treatment. We fitted a linear mixed model with the response variable as the standardised sperm number. The fixed effect is Treatment * Phase. Phase 1 is the mean of training trial value and phase 2 is the actual value. The random effect is male ID. We used drop1 function to drop out one term each time and ran anova between the model with and without that term.

```
lmer(Standardised sperm~Treatment*Phase+(1|Male.ID))
```

Since we tested the same general hypothesis four times. We therefore in addition to the 5% significance threshold, controlled for the risk of a false positive due to multiple comparisons using Bonferroni corrections⁴⁶. The new significance threshold was set at 1.25% (4 comparisons). We used an asterisk to signal the terms that were no longer significant after Bonferroni correction.

Results

Training trials

Firstly, in the training trials, males invested significantly more sperm in large-combed females (Table 1a, Fig. 2a). But there was no difference across treatments in terms of the probability of mating and probability of ejaculation. The latter suggests that males did not exert direct male choice (Table 1a, Fig. 2c). Instead, male choice mainly operates through cryptic male choice in terms of the standardised sperm number (Table 1, Fig. 2a).

The final trial

Secondly, in the final trial, males from the large treatment invested more sperm in the intermediate-combed female than males from the small treatment but this effect is not significant after Bonferroni correction (Table 1b, Fig. 2a).

Table 1. The analysis of variance (ANOVA) table

(a) lmer or glmer on the standardised sperm number, probability of ejaculation, probability of mating, and latency to ejaculate of the training phase

Response variable	Factors	df	Chisq	P value
Standardised sperm number	Treatment	1	11.152	<0.001
Probability of ejaculation	Treatment	1	4.548	0.033*
Probability of mating	Treatment	1	0.014	0.904
Latency to ejaculate	Treatment	1	0.416	0.519

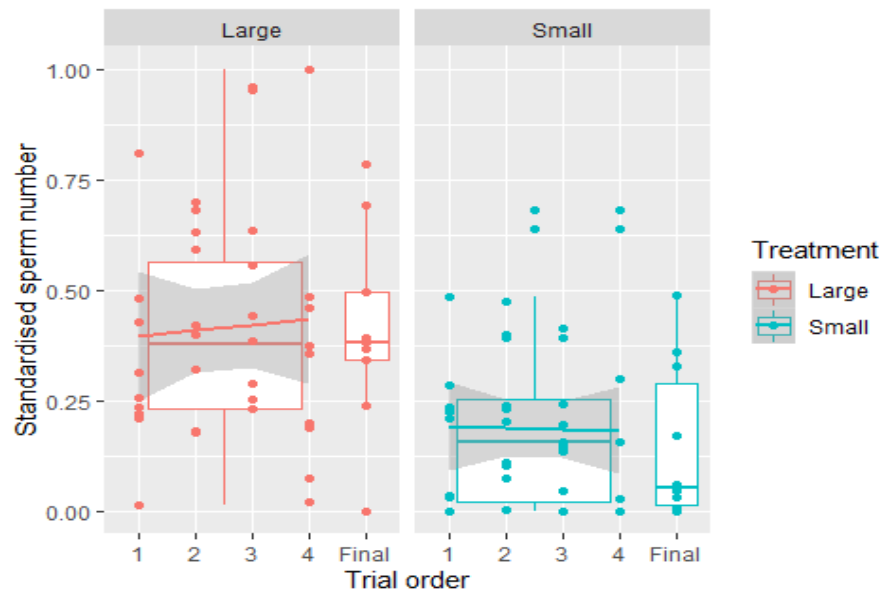
*no longer significant after Bonferroni correction (new significance threshold 1.25%)

(b) lmer or glmer on the standardised sperm number, probability of ejaculation, probability of mating, and latency to ejaculate of the final phase

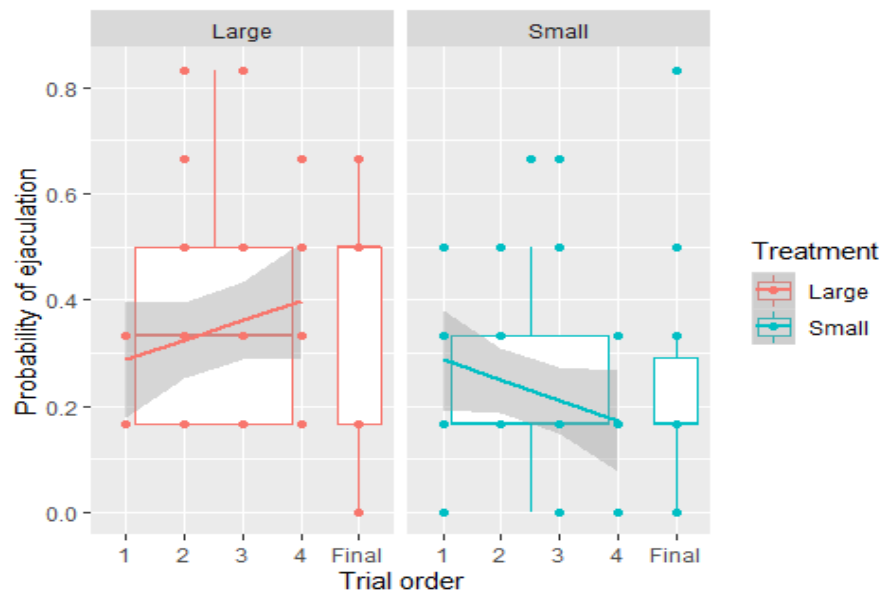
Response variable	Factors	df	Chisq	P value
Standardised sperm number	Treatment	1	7.642	0.013*
Probability of ejaculation	Treatment	1	0.992	0.319
Probability of mating	Treatment	1	0.873	0.35
Latency to ejaculate	Treatment	1	0.648	0.432

*no longer significant after Bonferroni correction (new significance threshold 1.25%)

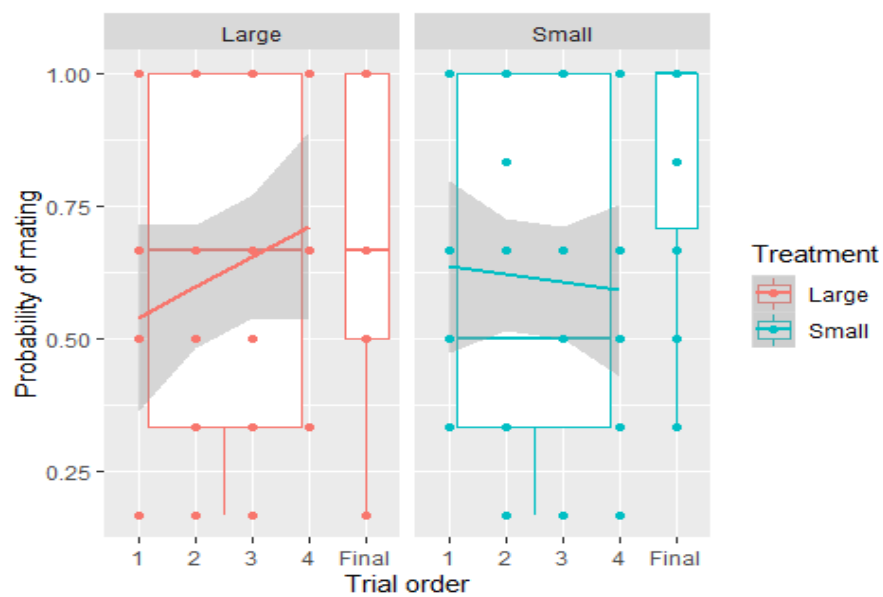
a



b



c



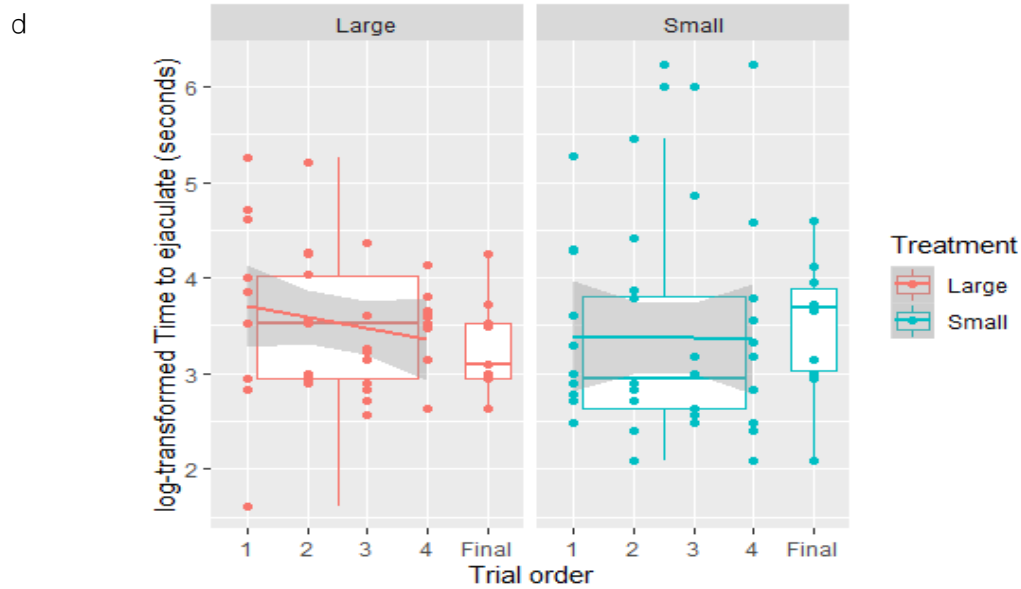


Figure 2. (a) The standardised sperm, (b) The probability of ejaculation, (c) The probability of mating in two treatments in the training and final phase and (d) The latency to ejaculate in two treatments in the training and final phase. Those are boxplots of the males in Treatment large and small. The boxplot shows the “minimum”, first quartile (Q1), median, third quartile (Q3), and “maximum”. On top of the box plot, there is a linear regression line with a 95% confidence interval of trial 1-4. On top of the regression line, there are jittered points. Each male contributes one point in each trial (i.e. 5 data points overall).

Predicted versus observed responses in the final trial

Treatment large males invest significantly more sperm than that from treatment small males, both in the training and final phases (Table 2, Fig. 3). Within each treatment, the training and final phase did not differ significantly (Table 2, Fig. 3).

Table 2. The analysis of variance (ANOVA) table

(i) *Method one: predicted final trial standardised sperm number vs actual final trial standardised sperm number*

Response variable	Factors	df	Chisq	P value
Predicted vs actual	Treatment*Phase	1	0.121	0.727
	Treatment	1	8.220	0.004
	Phase	1	0.498	0.480

(ii) *Method two: the mean of the standardised sperm number in the training trials vs actual final trial standardised sperm number*

Response variable	Factors	df	Chisq	P value
Training vs final	Treatment*Phase	1	0.224	0.636
	Treatment	1	10.148	0.001
	Phase	1	0.391	0.532

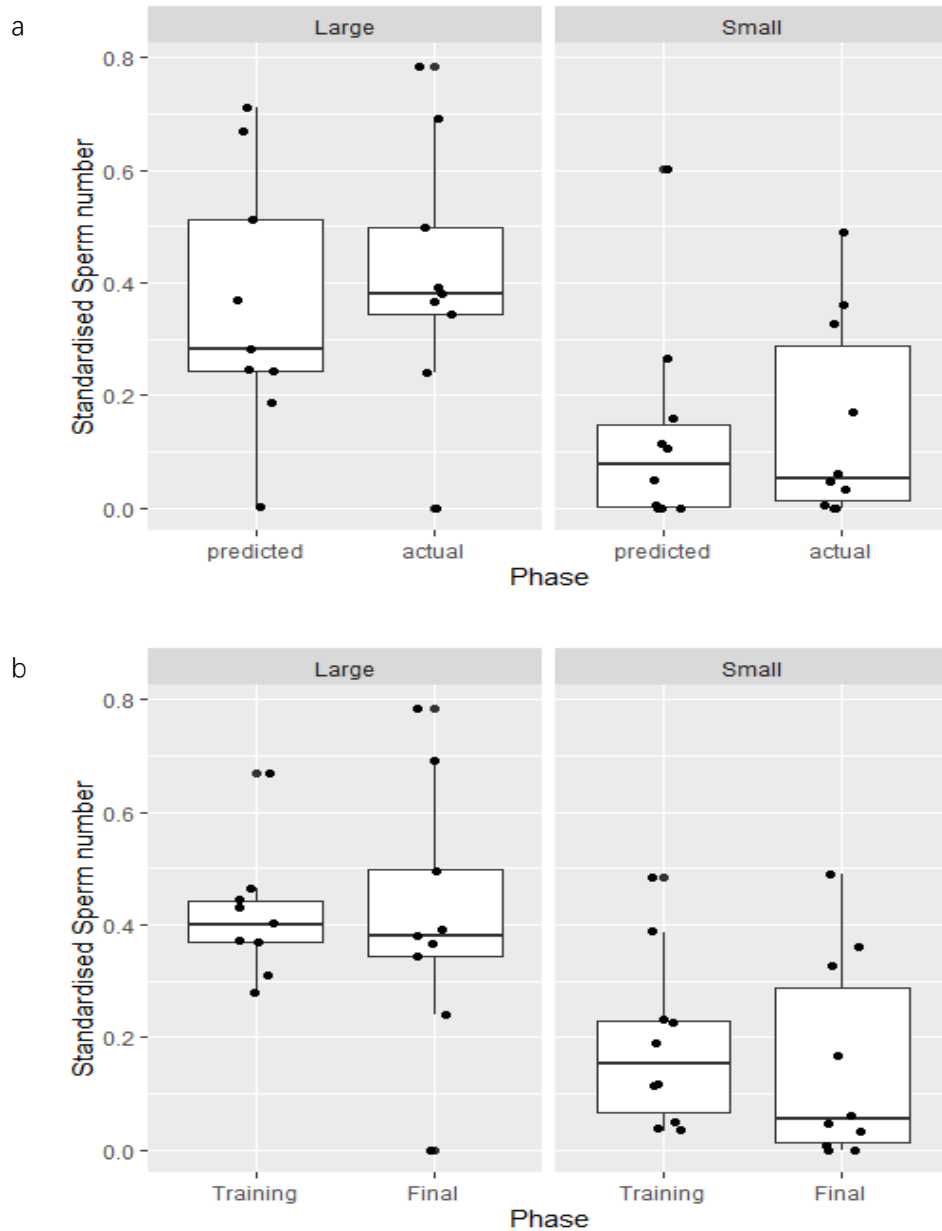


Figure 3. (a) Method 1: boxplot and jittered points of the standardised sperm number of the predicted values (generated from trial 1-4) and the actual final trial values in treatment large and small. (b) Method 2: boxplot and jittered points of the standardised sperm number of the mean of trial 1-4 and the actual final trial values in treatment large and small.

Discussion

This chapter investigates the mechanisms underpinning male choice of females. In both the training and final trials, males preferred females sporting a large ornament. However, males tend to exert the choice cryptically, i.e through differential sperm allocation, rather than directly. The results also showed that cryptic male choice was strongly influenced by previous experience. Males experienced a carry-over effect which meant that their sperm allocation to a female with intermediate comb size was predicted by the comb size of the females previously encountered by a male, rather than by the size of the comb of the current female. This was contrary to our expectation that males should adjust the sperm allocation in accordance with changes in female quality.

This experiment is novel in two main aspects. Firstly, this experiment follows a sequential paradigm. while most studies investigate male choice by presenting two females simultaneously. However, in natural conditions, it is very difficult for males to meet two receptive females at the same time. Sequential decision making is more complicated, because males need to make a decision not only based on the absolute female quality but also rely on experience and expectation for future mating. The major histocompatibility complex (MHC) is a class of genes that provides resistance to specific infectious diseases in vertebrates. Eggs can bias toward sperm with complementary MHC immunogens, resulting in an intermediate level of individual MHC diversity which confers highest pathogen resistance ⁴⁷. Male red junglefowl showed no preference when simultaneously presented with an MHC-similar and an MHC-dissimilar female in terms of the probability of mating. But when they sequentially mated with the two females, they invested significantly more sperm to the MHC-dissimilar female ⁴⁸. Secondly, this study examines the influence of recent experience in male choice decisions.

We postulated three predictions. Firstly, males would invest more sperm in large-combed females, than in small-combed females. Secondly, males who experienced

large-combed females in the training trials would decrease their sperm allocation in the final trial where they received an intermediate-quality female, while the males who experienced small-combed females will increase their sperm allocation in the final trial. Thirdly, intermediate-combed females will potentially get more sperm from males previously exposed to small-combed females in their training.

We found that males did invest more sperm in the large-combed females than in the small-combed females during the training trials. Edward and Chapman predicted that choosing higher-quality females yield net benefits for sperm-limited males ⁴⁹. Female red junglefowls with larger combs were significantly heavier than that with smaller combs and laid eggs that were significantly heavier ¹. Heavier chicks have a higher survival rate ⁵⁰. Hence, male choice towards higher-quality females might result in more viable offspring. In this experiment, males exerted male choice mainly by changing how many sperm to invest.

However, except for the first hypothesis, results were not consistent with our hypotheses. Males did invest more sperm in large-combed females when they encountered females sequentially. However, males did not vary sperm investment when exposed to a female of different quality in the final trial. Thirdly, intermediate-combed females received more sperm from males of large-combed females treatment. Males in the large-combed female group invested significantly more sperm and significantly more likely to invest sperm than the small-combed female group in the final trial. Carry-over effects represent cases where previous experience has strong bearing on current decision-making.

What is the evolutionary significance of this carry-over effect? Firstly, the carry-over effect might be adaptive. If male decision-making is costly and environments remain relatively stable. Males may gain by going on 'autopilot'. In the training trials female quality was quite constant and may have given the males confidence that the next

female would have been of similar quality. Secondly, the carry-over effect in male choice might be maladaptive. Males might be cognitively constrained to respond rapidly to environment fluctuation. To be able to respond to experimental procedures, males must acquire the ability to detect small differences in female comb size (i.e. difference in small-combed and intermediate-combed females). They might fail to spot the differences between the training trials and the final trial. This could be explored further by manifesting the differences between the training trials and the final trial (i.e. present 4 large-combed females in the training trial and 1 small-combed female in the final trial).

Similar carry-over effects in male choice have been found in other species. *Drosophila melanogaster* males are sensitive to the frequency distribution of mate quality, and display a mating preference for the phenotype of females with whom they previously mated, and which correspond to the most prevalent type in the population in the past. For example, males that experienced a preponderance of larger females had a preference for large females subsequently when a large- and small- female were presented simultaneously, while they preferred smaller females if they experienced a population dominated by small females and mated with a small female before ²⁹.

Male choice operates through two episodes. The first one is the direct male choice on whether to mate with this female or not. The second one is the cryptic male choice, which males strategically allocates a different amount of sperm and seminal fluid to different females. Mating and ejaculate expenditure may serve distinct functions. Mating itself benefits males. Firstly, in some species, mating can stimulate females to ovulate. Females that did not enter the breeding cycle may appear to be of low quality. It is a wastage of ejaculate if the female is not fertile. Mating may enable females to be more fertile. It was found that intromission without ejaculation is sufficient to induce female *Microtus ochrogaster* to ovulate ⁵¹. However, in red junglefowl, no such effect was documented. Secondly, copulation behaviour can serve for mate guarding purposes.

For instance, copulation in many species can reduce potential extra-pair copulation ⁵². Thirdly, mating may inhibit female from remating soon. Spermless copulation in the fly, *Bactrocera cucurbitae* inhibits female remating ⁵³. In the red junglefowl, mating alone reduced female remating propensity ⁵⁴. Fourthly, mating or intromission without ejaculation can facilitate the formation of a pair bond. In voles, intromission provides a signal to release oxytocin which plays a key role in pair bonding ⁵⁵. Fifthly, in some species, females copy other females, especially older females' mate choice. A male might become popular if he is able to mate with multiple females even though he does not have enough sperm to fertilise every female. For instance, the mate choice of female guppy, *Poecilia reticulata*, can be reversed if the unfavoured male mates with other females ⁵⁶. Sixth, intromission may help to remove the copulation plug from the previous male. For example, lab rat intromission can remove the sperm or copulation plug in the female reproductive tract left by previous males ⁵⁷. However, there is no intromission organ in fowls.

For red junglefowl, even though males do not possess a penis to effectively remove sperm left by previous males, copulation behaviour can serve to reduce the levels of sperm competition. On the female side, mounting alone can reduce female's propensity to remate in red junglefowl ⁵⁴. Thus the male will face a lower risk of future sperm competition. On the male side, males prefer to mate with females with lower levels of sperm competition ⁹. Rival males are less likely to mate with a female that has mated recently. If the rival male observes the mating scene, he will be less likely to mate with this female thus reducing the levels of future sperm competition. Therefore, it is important to disentangle the direct and cryptic male choice in the red junglefowl.

Conclusion

Male red junglefowls have the ability to assess female quality based on the expression of a female ornament, the comb, and male choice operates mainly cryptically through differential sperm allocation. Males prefer large-combed females under sequential

encounters. Following exposure to consistently large- or small-combed females appeared to influence male sperm allocation to a female of intermediate comb size in the final trial, so that the intermediate females received more sperm from males previously exposed to large-combed females. This carry-over effect might be an adaptive way to reduce the costs of decision-making when social environment is relatively stable or might reflect physiological and cognitive constraints.

Appendix

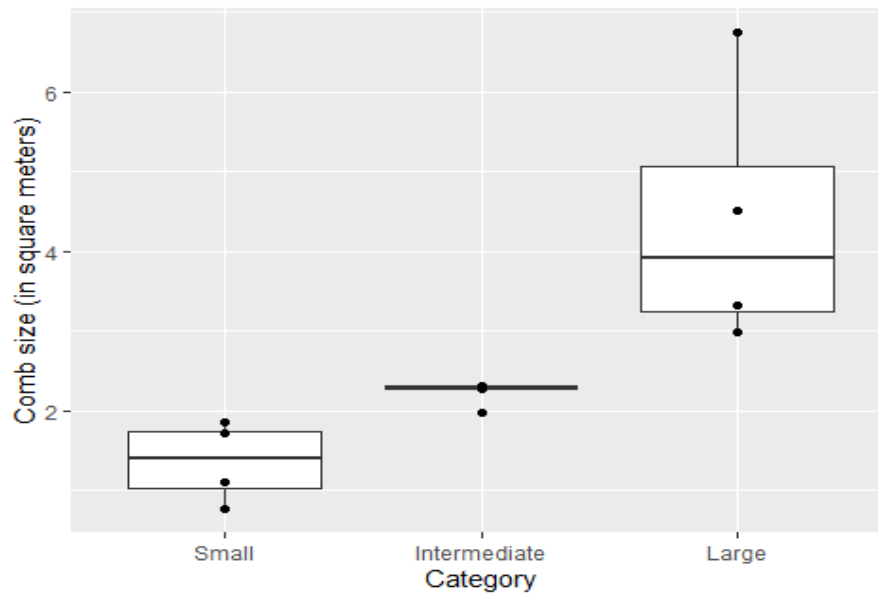


Figure A1. Female comb size in cm^2 of low-, intermediate, and large-comb groups. This is a boxplot of the female com size in three groups. It shows the “minimum”, first quartile (Q1), median, third quartile (Q3), and “maximum”. Superimposed on the box plot are jittered points. Each female contributes one point in each trial.

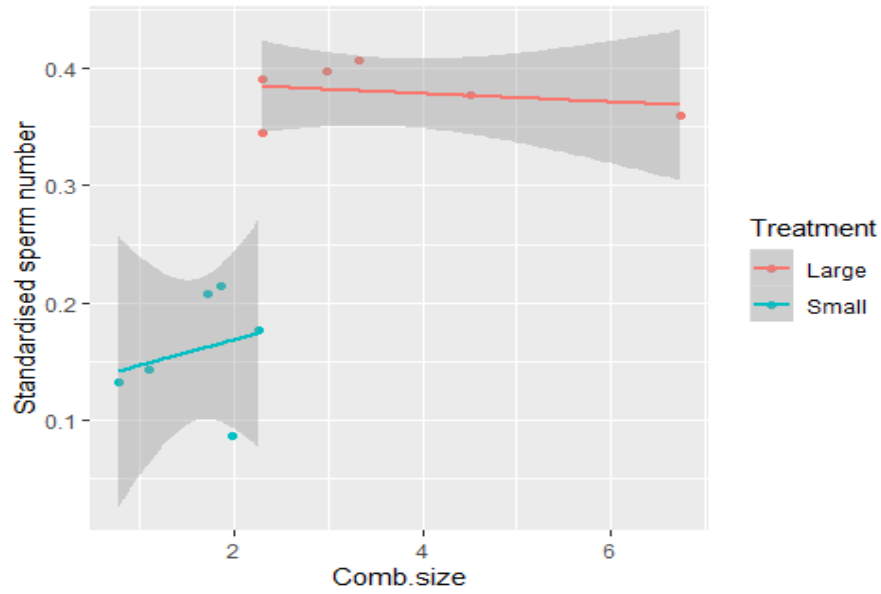


Figure A2. Linear regression line of standardised sperm number over comb size with 95% CI in treatment small and large. Within each treatment, the comb size was not a significant predictor (Large: $\chi^2 = 0.436$, $P = 0.509$, Small: $\chi^2 = 0.871$, $P = 0.351$).

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Chapter 5: The Influence of Past Experience on Male Investment in a Novel Female

Abstract

Sperm production is costly and the depletion of sperm reserves can limit male reproductive success. In polygynandrous mating systems, males must balance investing sufficient sperm in the current partner in order to outcompete the ejaculates of other males while saving sperm for new mates. Often males encounter females sequentially and thus a male must decide how much to invest in the current female and how much to save for new females he may encounter in the near future. In some species males might solve this problem by progressively reducing ejaculate expenditure in the current female and increasing it upon encountering a new female, a preferential investment in sexually novel females, known as the Coolidge effect. While patterns of ejaculate expenditure consistent with the Coolidge effect have been described in some polygynandrous species, the causal role of female novelty has not been experimentally demonstrated. Furthermore, saving sperm for novel females requires an expectation of future mating opportunities, suggesting that prior experience may mediate male decision-making process under sequential encounters. Here, we build on previous work on the Coolidge effect in male red junglefowl and experimentally tested whether: (i) female sexual novelty changes male ejaculate expenditure, (ii) males respond to different probabilities of encountering novel females, and (iii) past experience informs male ejaculate expenditure. We found that males reduced ejaculate expenditure as a female became more familiar and increased it when exposed to a novel female compared to control treatments, demonstrating the causal effect of female novelty. Secondly, males were sensitive to the probability of encountering a novel female and reduced ejaculate expenditure under uncertainty though the results are not significant after Bonferroni correction. Finally, we found some evidence that past experience might influence ejaculate expenditure strategies: ejaculate expenditure was similar in the first trial across treatments, when males were still naïve about the different regimes of

female availability imposed by different treatments, but tended to diversify in subsequent stages of the experiment when males had acquired more information. However, the effects are not significant. Collectively, these results demonstrate that patterns of female novelty play a key role in the evolution of male ejaculate expenditure strategies in polygynandrous species.

Introduction

Bateman argued that male reproductive success is positively related to male mating success (i.e. the number of females that a male inseminates), while female reproductive success is independent on the number of mates since fertilisation of a set of eggs is typically guaranteed by one or few copulations¹. This asymmetry can be captured by the Bateman gradient, the slope of the regression of reproductive success over mating success, which measures the strength of sexual selection on mating success. In many species, the Bateman gradient is steeper in males than in females². Importantly, in sex-role reversed species, the Bateman gradient is steeper in females than in males. For example, in the sex-role reversed dusky pipefish, *Syngnathus floridae*³, indicating that when ecological conditions change sex-specific constraints on potential reproductive rates, sex differences in sexual selection for mating success change accordingly. These patterns confirm Bateman's prediction that male reproductive success is typically limited by the availability of fertilisation opportunities, resulting in strong sexual selection on males to increase their mating success⁴.

This prediction however does not fully capture the costs of ejaculate expenditure⁵. Recent studies show that ejaculate is costly and limiting. The depletion of sperm reserves through mating can limit male reproductive success. These are particularly conspicuous in polygynandrous mating systems, where males have the opportunity to mate with multiple females, but females mate with multiple males generating sperm competition. Sperm competition occurs when sperm from different males competes for the same set of ova, and generates post-copulatory sexual selection on males to increase ejaculate expenditure in individual females in order to maximise their share of paternity. Thus, under polygynandry male reproductive success is dependent on how many females a male inseminates (mating success) and his ejaculate expenditure in a female relative to that of other competitors inseminating the same female⁵. Males are therefore expected to maximise reproductive success through strategies that optimise ejaculate expenditure across different mating opportunities⁶.

Achieving this however, is non-trivial. Often in nature males encounter females sequentially and thus a male must decide how much to invest in the current female and how much to save for new females he may encounter in the near future (i.e. before the sperm invested in current mating is replenished). A strategy through which males may potentially address this is by preferentially investing based on the sexual novelty of a mating partner. In several species, male sexual interest in a female declines the more a male mates with the female, and peaks again in the presence of a sexually novel female, which enables males to modulate sperm investment in successive females. This pattern is known as the Coolidge effect, which refers to a progressive decline in a male's propensity to mate with the same female over time, combined with a heightened sexual interest in a novel female ⁷. The Coolidge effect suggests that males have a propensity to reserve some sperm for a potential future new mate. Patterns consistent with the Coolidge effect have been described in several species ^{7, 8-11}. For example, a study of a simultaneous hermaphrodite, the pond snails, *Lymnaea stagnalis*, provided direct evidence for higher mating propensity when a new partner is encountered ¹². The Coolidge effect might also differ according to the male's social status. European bitterling, *Rhodeus amarus*, is an externally fertilising species. Dominant males invested more sperm in the novel females, while subordinate males were not influenced by female's novelty. The different response of dominant and subordinate males suggests males can allocate sperm strategically based on their likelihood of encountering a new female ¹³. However, some species, mostly monogamous species show no Coolidge effect, and the absence of a Coolidge effect has been treated as an indicator of monogamy. For example, no Coolidge effect was found in the monogamous prairie voles, *Microtus ochrogaster* ¹⁴. In species where males invest highly in a mating, sex-roles and the Coolidge effect might be reversed. For example, in the polyandrous decorated crickets, *Gryllodes sigillatus*, where males produce a large spermatophore, female competition over males is strong, and females prefer sexually novel males while males do not prefer novel females ¹⁵.

The Coolidge effect is a sequential decision-making process, which requires a male exercising a level of self-restraint with the current female to save sperm for females that may become available in the near future. Decision-making under a sequential encounter is more complex than a simultaneous encounter because the former involves generating an expectation of future outcomes and temporal discounting, which is the process of systematically devaluating outcomes over time. In an uncertain world, animals face both unexpected opportunities and costs. Selection should favour adaptations to mitigate the potential costs of uncertainty. Dall (2002) argued that animals can buffer themselves against uncertainty by storing food or fat when future feeding is uncertain to avoid starvation in the future^{16,17}. Very little however, is known about sequential decision-making in the context of sexual selection and ejaculate expenditure strategies. Galvani & Johnstone (1998) proposed models of optimal sperm allocation using stochastic dynamic programming techniques¹⁷. These models predict that when males mate sequentially while sperm stores are limited, they should prefer to withhold sperm when the future mating opportunity is uncertain. For example, males should withhold sperm early in the season in case a high-quality female or a novel female is encountered later¹⁸.

Animals may rely on the recent experience of exploring environments and learning the probabilities and outcomes associated with each option to make a decision¹⁹. For example, individuals may examine a pool of potential mates, compare current opportunities with previous opportunities, and choose the best of n based on experience²⁰. In the three-spined sticklebacks, *Gasterosteus aculeatus*, females choose mates based on comparison with previous males²¹. In addition, past sexual experience may influence current decision-making by mediating homeostatic responses, such as strong physiological changes in heart rate, blood pressure, electromyogram, cortisol level, skin temperature, and skin conductance response²². Prior experience may associate with a homeostatic change, which subsequently affects decision-making. For example, neural

activation may reflect the homeostatic changes associated with different risk experiences. Individual participants experienced low-risk task significantly increased the urge for taking a risk subsequently²³. Under sequential mate encounters, males may base their decisions on how much to invest in current mating opportunities based on previous experience to decide when to stop mating with the current female in order to save sperm for a new female. This entails males estimating the probability of encountering a new female based on past experience. Information required by ejaculate expenditure decisions might then be built up over time²⁴. Most studies of male choice however, adopt a simultaneous choice paradigm, and few have explicitly investigated male decision-making under sequential mate encounters. Thus, while there is increasing evidence that males allocate sperm differentially in response to socio-sexual conditions associated with different copulations in a way that is broadly consistent with theory (see^{25 26} reviews), little remains known about how experience informs male ejaculate expenditure decisions²⁵.

In this chapter, we explored male ejaculate expenditure in response to female novelty in the polygynandrous red junglefowl, *Gallus gallus*. The red junglefowl is a Phasianid²⁷ which and the main ancestor of the domestic fowl, *G. domesticus*²⁸. Males from some populations of red junglefowl and domestic fowl display sophisticated ejaculate expenditure strategies, through which males modulate sperm allocation in response to the level of sperm competition²⁹, the reproductive quality of a female^{29,30}, their genetic similarity with a female^{31,32}. Importantly, males show differential patterns of sexual interest and ejaculate expenditure in response to female novelty which consistent with the Coolidge effect. A male's propensity to mate with a female and the number of sperm allocated to an insemination decreased logarithmically over successive exposures of a male to a female, and increased when the sexually familiar female was replaced by a sexually novel female²⁹. A more recent study confirmed these patterns and found that sperm number, seminal fluid volume, and seminal fluid proteins concentration decreased as the male mated with the same female and rose when the male mated with

a novel female ³³. The seminal fluid allocated to a sexually novel female also had a distinct proteomic signature compared to that allocated to the first female, suggesting that differential ejaculate expenditure strategies in relation to female novelty may also involve differential investment in individual seminal fluid proteins ³³. This work however, lacked experimental controls, and thus cannot rule out that these patterns may be driven by successive matings *per se* (i.e. a mating order effect), rather than by the exposure to a novel female in the last matings of mating sequence.

Aims

Here, we used an experimental protocol to answer three aims.

1. To test male preferential expenditure in a sexually novel female controlling for mating order.
2. To test whether male ejaculate expenditure is influenced by uncertainty.
3. To investigate how past experience influences ejaculate expenditure patterns.

Methods and materials

We studied a population of red junglefowl at the John Krebs's field station of the University of Oxford in Wytham, during the breeding season 2017 (July-October) and 2018 (April-July). We selected 24 sexually mature healthy males and assigned 8 to each of 3 training treatments. In treatment 1 (T1), a male was exposed to one female in each trial, and could mate up to 6 times with this female within 60 minutes. In treatment 2 (T2), a male was exposed to two females sequentially in each trial. A male could mate up to 3 times within 30 minutes with the first female, following which the now sexually familiar female was replaced by a sexually novel female and the male had the opportunity to mate up to 3 times within 30 minutes with this second female. In treatment 3 (T3), males were exposed to a trial identical to T1 (with one female) or to T2 (with two females sequentially), in random order with an overall probability of 0.5 that the first female would be replaced by a second female on the 4th mating opportunity of a trial.

Prior to the experiments, males were kept in single-sex pens of around 12 birds. All males used in the experiments were non-virgins and they were sexually rested for at least 48 hours. During the experiment, each male was housed in an individual pen, visually but not vocally isolated from other birds, and was exposed to an experimental trial every other day to ensure replenishment of extragonadal sperm reserves ³⁴, under the regulation of the project license (PPL: P50402706). The training phase comprised of 13 to 14 training trials for each male for all treatments (T1, T2, T3). Males were of similar age across treatments (T1: 4.5 ± 0.53 , T2: 3.9 ± 0.40 , T3: 4.4 ± 0.42). We used a cohort of females of similar comb length (randomly selected from a pool of females with similar comb length, 30.7 ± 0.50 mm, $n=24$). A set of six females was used in the training trials of T2 and the T3 trials using two females. A set of three females was used in the T1 training trials. Thus, males encountered the same female again every three trials. While this means that a female was not completely novel to a male, we assumed that a separation of six days would be sufficient to make the female sexually novel to

the male. In the final trial, males from all treatments were exposed to the same set of two novel females, and were given an opportunity to mate up to 3 times in 30 minutes with the first female, following which the first female was replaced with the second female and the male was given the opportunity to mate up to 3 times in 30 minutes with this second female. Thus, the final trial was identical in design to T2 trials. Here is the experimental diagram (Fig. 1).

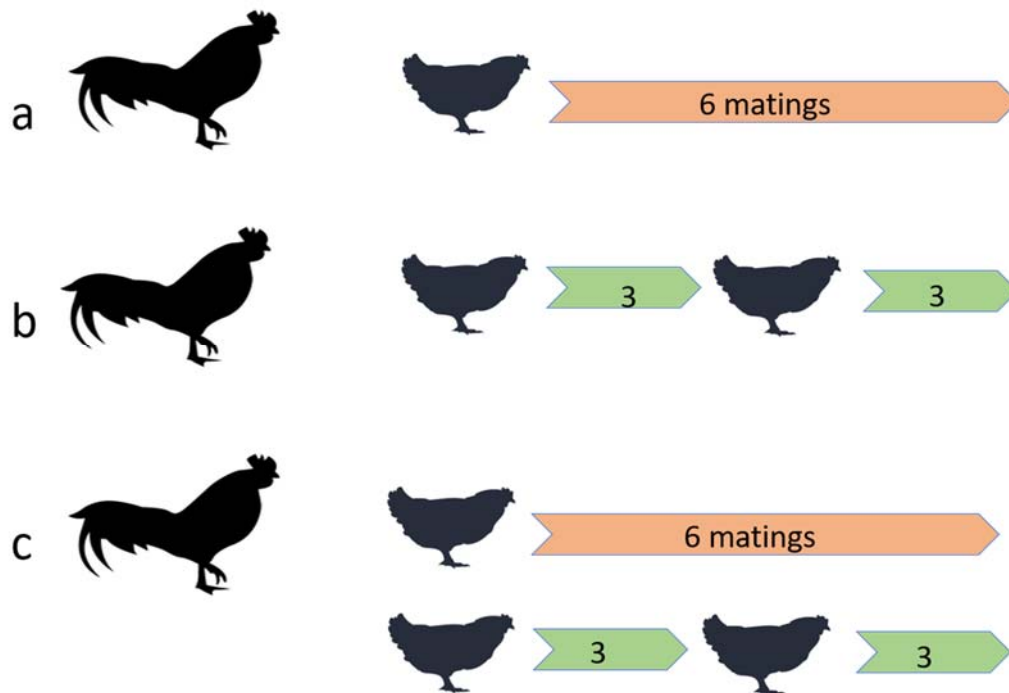


Figure 1. (a) T1 – trained with always one female. (b) T2 – trained with always two females. (c) T3 – trained with one or two females in random order.

We measured ejaculate expenditure using a well-established protocol based on the experimental exposure of a male to a female fitted with a harness covering her cloaca³⁵. A female was held for one minute facing the focal male in order to enable the male to inspect her. Then the female was turned around and held in a soliciting position, placed on a plastic substrate and with a harness covering her cloaca to facilitate ejaculate collection by preventing contact with the female reproductive tract. We recorded the time to ejaculate, which is the time elapsed from presenting the female in a soliciting position to the first successful ejaculate. We terminated the trial if the male

did not mate for 15 minutes. After each copulation, we collected ejaculates into individual Eppendorf tubes using a Gilson pipette. We diluted 2.5 μ l of semen into 197.5 μ l of Phosphate-buffered saline (PBS) and transferred 70 μ l of the mixture to a spectrophotometer set at 595nm to measure the concentration of sperm within the ejaculate through the light absorbance. We used a standard curve to calculate sperm concentration and measured the volume of the ejaculate with a Gilson pipette. We used the sperm concentration and ejaculate volume to estimate the number of sperm contained in the ejaculate. More details of this protocol can be found in chapters 2.

Statistical analyses

All analyses were conducted using the statistical software R with the lme4, lsmeans, dplyr, ggplot2, car packages.

Aim 1. To test male preferential expenditure in a sexually novel female controlling for mating order

(i) *Comparing Treatment 1 with Treatment 2*

We first tested this by comparing ejaculate expenditure measures in the training stage between treatment 1 and 2. Thus, we investigated mating behaviour over the sequence of 6 mating opportunities when the first female was replaced by a second at the 4th mating opportunity (T2) and compared it with the behaviour of males in the control T1 where males were exposed to the same female across the 6 mating opportunities of a trial. We tested whether the probability of mating differed across the two treatments. To do this, we fitted a generalised linear mixed effect model to the data with a binomial error structure, with the successful copulation as the response variable (a successful copulation as “1”, no copulation as “0”). We created a fixed effect (Female order) that captured the first half of a trial (first 3 mating opportunities, Female order = 1) and the second half (Female order = 2), and a fixed effect (copulation) to capture mating order (1st, 2nd, 3rd) within each half of the trial. We included treatment (T1 vs T2), Female order (1 vs 2) and the copulation (from 1st to 3rd) and their 2- and 3-way interactions as fixed effects, and male ID, female ID, trial number (1 to 13 or 14) as random effects. We dropped out one term each time and tested the significance of each term by comparing the model with and without that term using anova type II analysis (Chisq). Chi-square distributions have only one tail and therefore the significance values were one-tailed.

```
glmer(Mate or Not ~Treatment*Copulation.order*Female.order+(1|Male.ID)+  
(1|Female.ID)+(1|Trial),family=binomial)
```

We then tested whether the probability of ejaculation differs across T1 and T2, excluding trials where the male did not mate at all. We fitted the same generalised linear mixed effect model, with the probability of ejaculation as the response variable (a successful ejaculate as “1”, aspermic copulation as “0”), while others remain the same. We dropped out one term each time and tested the significance of each term by comparing the model with and without that term using anova type II analysis (Chisq).

```
glmer(Ejaculate or not ~Treatment*Copulation.order*Female.order + (1|Male.ID)
+(1|Female.ID) +(1|Trial), family=binomial)
```

Similarly, we excluded data points where the male did not mate, and we tested whether the latency to ejaculate differs across the two treatments. We fitted a linear mixed effect model, with $\log_2(\text{time} + 1)$ as the response variable, while others remain the same. We log-transformed the response variable to meet the normality assumption. We added 1 to the time because some males ejaculated immediately as the female was put in a soliciting position so the time counted as zero. Afterwards, we dropped out one term each time and tested the significance of each term by comparing the model with and without that term using anova type II analysis (Chisq).

```
lmer(log2(Time+1)~Treatment*Copulation.order*Female.order+(1|Male.ID)+(1|Female.ID)+(1|Trial))
```

Finally, we excluded data points where the male did not deliver sperm, and analysed variation in sperm numbers. We standardised the number of sperm estimated for each ejaculate by dividing it by the largest ejaculate produced by the same male within the 6 mating opportunities of a single trial. We fitted a generalised linear mixed effect model, built as above with standardised number as the response variable. We dropped out one term each time and tested the significance of each term by comparing the model with and without that term using anova type II analysis (Chisq).

```
glmer(Standardised.sperm~Treatment*Copulation.order*Female.order+(1|Male.ID)+(1|Female.ID)+(1|Trial),family=binomial)
```

(ii) *Comparing one female (T3.1) with two females (T3.2) subgroup in Treatment 3*

In addition, we further tested preferential expenditure in sexually novel females by comparing the response of individual males when exposed to one or two females across different trials in Treatment 3. We broke down T3 into two balanced subgroups, one-female subgroup (control group) and two-female subgroup. We conducted the same statistical analyses as above.

Aim 2. To test whether male ejaculate expenditure is influenced by uncertainty

(i) *Comparing Treatment 1 with Treatment 3.1*

We first compared male behaviour in Treatment 1 and in the one-female trial of in Treatment 3. We tested whether the probability of mating differs across Treatment 1 and one-female trial subgroup in Treatment 3. To do this, we fitted a generalised linear mixed effect model to the data with a binomial error structure, with the successful copulation as the response variable (a successful copulation as “1”, no copulation as “0”), treatment, Female order and copulation (from 1 to 3) and their interactions as fixed effects, and male ID, female ID, trial number as random effects. We dropped out one term each time and tested the significance of each term by comparing the model with and without that term using anova type II analysis (Chisq).

```
glmer(Mate or not ~Treatment or Subgroup*Copulation.order*Female.order  
+(1|Male.ID)+(1|Female.ID)+(1|Trial),family=binomial)
```

We excluded data points where the male did not mate, and analysed variation in the probability of ejaculation. We fitted the same generalised linear mixed effect model as above, and dropped out one term each time and tested the significance of each term by comparing the model with and without that term using anova type II analysis (Chisq).

```
glmer(Ejaculate or not~Treatment or Subgroup*Copulation.order*Female.order
```

$+(1|\text{Male.ID})+(1|\text{Female.ID})+(1|\text{Trial})$, family=binomial)

We also tested variation in latency to ejaculate. We fitted a linear mixed effect model and dropped out one term each time and tested the significance of each term by comparing the model with and without that term using anova type II analysis (Chisq).

$\text{lmer}(\log_2(\text{Time}+1) \sim \text{Treatment} \quad \text{or} \quad \text{Subgroup} * \text{Copulation.order} * \text{Female.order} + (1|\text{Male.ID}) + (1|\text{Female.ID}) + (1|\text{Trial}))$

We further excluded data points where the male did not deliver sperm, and tested whether standardised sperm differs across two subgroups. We fitted a generalised linear mixed effect model and dropped out one term each time and tested the significance of each term by comparing the model with and without that term using anova type II analysis (Chisq).

$\text{glmer}(\text{Standardised.sperm} \sim \text{Treatment} \quad \text{or} \quad \text{Subgroup} * \text{Copulation.order} * \text{Female.order} + (1|\text{Male.ID}) + (1|\text{Female.ID}) + (1|\text{Trial}), \text{family} = \text{binomial})$

(ii) Comparing Treatment 2 with Treatment 3.2

We then used the same approach to compare treatment 2 with the two-female trial subgroup of treatment 3.

Aim 3. To investigate how past experience influences ejaculate expenditure patterns

We compared male behaviour across three stages: (i) the first of the training trials, (ii) the rest of the training trials, and (iii) the final trial. In the first trial, males are naïve to the experimental design and have no information about how many mating opportunities they will have and with how many females. In trial 2-14 of the training stage, males become progressively more informed about the regime of female availability simulated by each treatment. In the final trial, T2 males will be familiar with the protocol being

identical to all previous trials, T3 males will be partially familiar with the protocol being identical to half of their previous trials, while T1 males will be unfamiliar having been trained to expect only one female in a trial. We focused on the 4th mating opportunity of a trial as diagnostic of male expenditure as this corresponds to the point in a trial when a female is replaced by a novel female (T2, two-female trials in T3) or not (T1, one-female trials in T3). For stage (ii), we computed the mean response in the 4th mating across trials 2-14 for each male. We fitted one generalised linear mixed model using binomial family. The response variable was standardised sperm number. Fixed effects were stage ((i), (ii), (iii)), Treatment and their interactions. Male ID was entered as a random effect. We dropped out one term each time and tested the significance of each term by comparing the model with and without that term using anova type II analysis (Chisq).

```
glmer(Standardised sperm~Stage*Treatment+(1|Male.ID), family=binomial)
```

We fitted another linear mixed model for latency to ejaculate. The response variable is $\log_2(\text{Time} + 1)$. The fixed effect is stage * Treatment. The random effect is male ID. We dropped out one term each time and tested the significance of each term by comparing the model with and without that term using anova type II analysis (Chisq).

```
lmer(log2(Time+1)~Stage*Treatment+(1|Male.ID))
```

In aims 1 and 2, we tested the same general hypothesis four times (probability of mating, probability of ejaculating, standardised sperm number and latency to ejaculate). We therefore controlled for the risk of a false positive due to multiple comparisons using Bonferroni corrections³⁶. Thus, for the analysis the significance threshold was set at 1.25% (5% divided by 4 comparisons). For aim 3, we tested the same hypothesis twice (Standardised sperm and latency to ejaculate). Thus, we set the significance threshold at 2.5%. We used an asterisk to signal the terms that were no longer significant after Bonferroni correction.

Results

Aim 1. To test male preferential expenditure in a sexually novel female controlling for mating order

(i) Comparing Treatment 1 with Treatment 2

General patterns indicated an overall decline in expenditure over the course of the 6 mating opportunities across training trials represented by a significant Female order by Copulation order interaction (i.e. lower probability of mating, lower probability of ejaculation, longer latency to ejaculate and fewer sperm, Table 1, Fig. 2), suggesting a consistent effect of depletion. However, there were important differences across treatments.

Comparing Treatment 1 and Treatment 2, there was a significant Treatment by Female order and Treatment by Copulation effects for the probability of mating, indicating that males had a significantly higher probability of mating in the second half of a trial in T2, where they were provided with a second female, compared to males in T1, which were still exposed to the first female (Table 1, Fig. 2a).

There was no significant Treatment by Female order effect for the probability of ejaculation, and a marginally significant effect of Treatment by Copulation. The latter effect lost significance after Bonferroni correction although males appeared more likely to deliver sperm in the 4th mating of T2 compared to T1 (Table 1, Fig. 2b).

There was a weak but significant effect of Treatment by Female Order for standardised number of sperm delivered, whereby T2 males allocated proportionally more sperm to copulations in the second half of the trial compared to T1 males (Table 1. Fig. 2c), which indicates that males allocate more sperm to the novel female than the familiar female. However, this effect lost significance after Bonferroni correction.

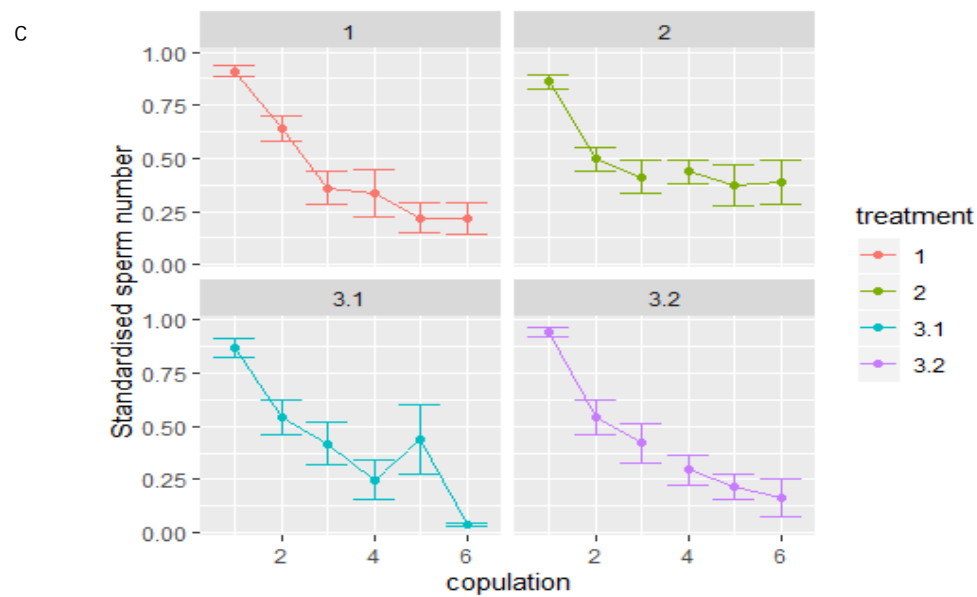
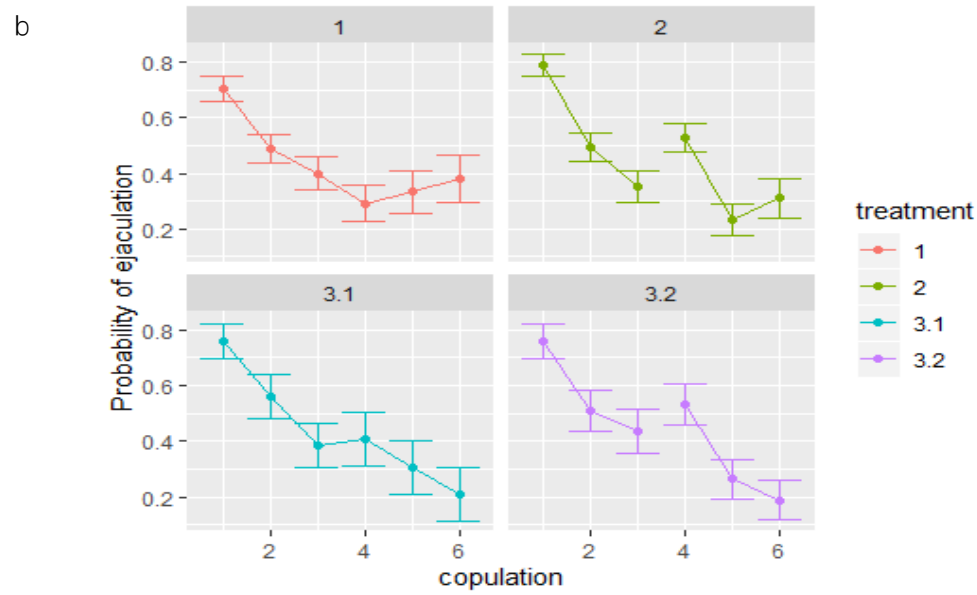
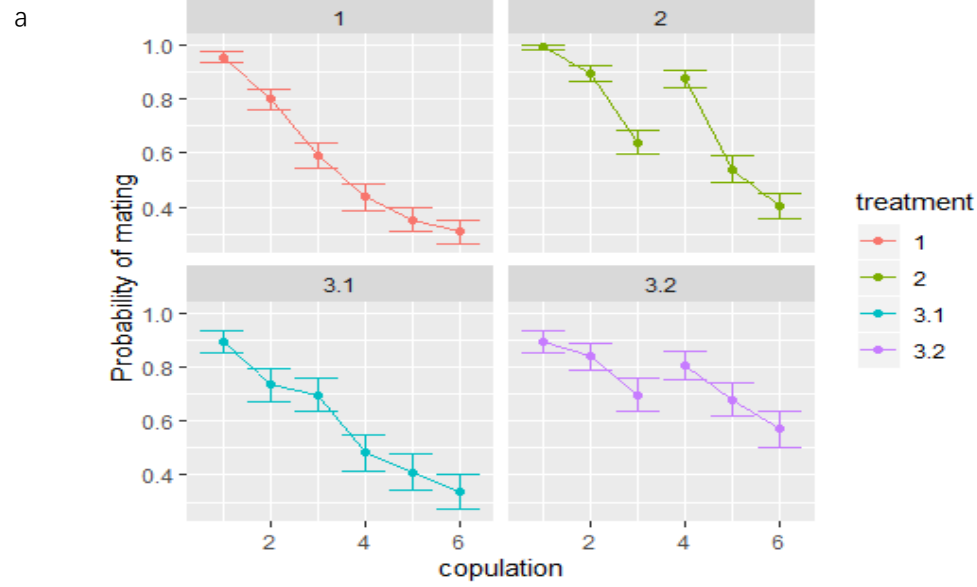
There was a significant Treatment by Copulation order effect for the latency to ejaculate (Table 1, Fig. 2d), whereby T2 males were faster to mate in the second half of the trial (particularly the 4th copulation) compared to T1 males, which indicates that males were faster to mate with a novel female than a familiar female.

Table 1. The analysis of variance (ANOVA) table:

glmer or lmer on the probability of mating, probability of ejaculation, standardised sperm number and latency to ejaculate of the training trials of Treatment 1 and Treatment 2				
Response variable	Factors	df	Chisq	P value
Training trials of Treatment 1&2				
Probability of mating	Treatment*Copulation*Female.order	1	1.652	0.199
	Treatment*Copulation	1	8.320	0.004
	Treatment*Female.order	1	8.113	0.004
	Copulation*Female.order	1	23.216	<0.001
	Treatment	1	1.871	0.171
	Female.order	1	230.47	<0.001
	Copulation	1	166.44	<0.001
Probability of ejaculation	Treatment*Copulation*Female.order	1	1.503	0.220
	Treatment*Copulation	1	5.013	0.025*
	Treatment*Female.order	1	0.834	0.361
	Copulation*Female.order	1	10.215	0.001
	Treatment	1	0.434	0.510
	Female.order	1	42.973	<0.001
	Copulation	1	50.877	<0.001
Standardised sperm number	Treatment*Copulation*Female.order	1	0.201	0.654
	Treatment*Copulation	1	1.913	0.167

		Treatment*Female.order	1	4.965	0.026*
		Copulation*Female.order	1	8.228	0.004
		Treatment	1	0.308	0.579
		Female.order	1	68.913	<0.001
		Copulation	1	65.97	<0.001
Latency to ejaculate		Treatment*Copulation*Female.order	1	0.501	0.479
		Treatment*Copulation	1	19.597	<0.001
		Treatment*Female.order	1	0.438	0.508
		Copulation*Female.order	1	1.739	0.187
		Treatment	1	0.843	0.358
		Female.order	1	86.53	<0.001
		Copulation	1	110.14	<0.001

* no longer significant after Bonferroni correction (new significance threshold 1.25%)



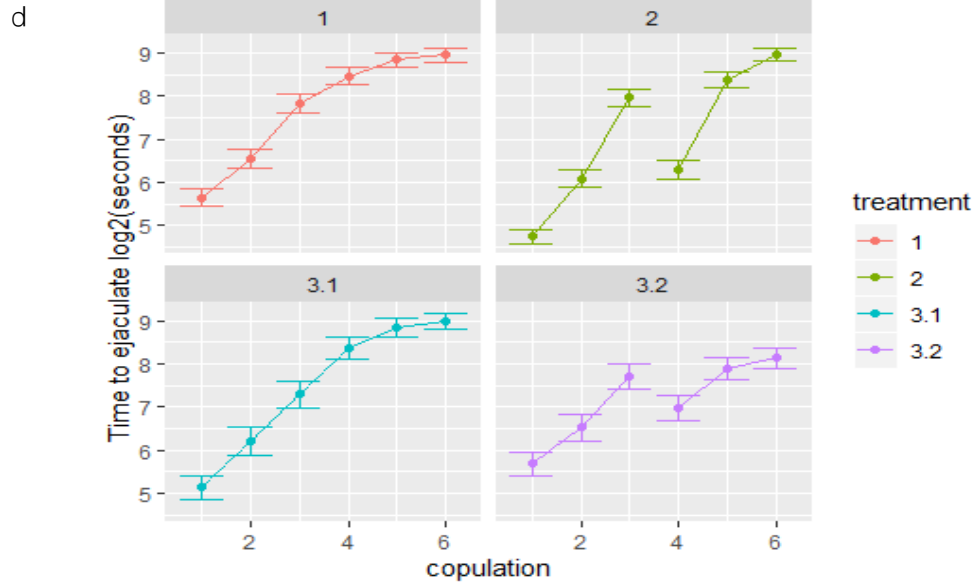


Figure 2. (a) Probability of mating, (b) probability of ejaculation, (c) standardised sperm numbers, and (d) latency to ejaculate ($\log_2(\text{Time} + 1)$) over successive mating opportunities in Treatment 1 (1), Treatment 2 (2), and in the one- and two-female trials of Treatment 3 (3.1, 3.2, respectively). Data points represent means of all males in each treatment. Vertical bars = SE.

(ii) *Comparing Treatment 3.1 with Treatment 3.2*

We found a similar but less pronounced preferential investment in novel females when comparing a male's behaviour in one- versus two- female trials in Treatment 3. The probability of mating in the second half of a trial was significantly lower in one female sub-treatment than in the two female sub-treatment as reflected by a significant Treatment by Female order interaction (Table 2, Fig. 2a). There was a significant Treatment effect also, indicating males were more prone to mate with the novel females (Table 2, Fig. 2a).

There was no Treatment effect in terms of the probability of ejaculation or in standardised sperm number (Table 2, Fig. 2b, c). However, there was a strong Treatment by Female order interaction effect in terms of latency to ejaculate, which indicated a heightened sexual interest towards the novel female (Table 2, Fig. 2d).

Table 2. The analysis of variance (ANOVA) table:

glmer or lmer on the probability of mating, probability of ejaculation, standardised sperm number and latency to ejaculate of the training trials of Treatment 3				
Response variable	Factors	df	Chisq	P value
Training trials of Treatment 3				
Probability of mating	Treatment*Copulation*Female.order	1	<0.001	0.98
	Treatment*Copulation	1	0.216	0.642
	Treatment*Female.order	1	17.448	<0.001
	Copulation*Female.order	1	1.76	0.185
	Treatment	1	17.419	<0.001
	Female.order	1	91.368	<0.001
	Copulation	1	44.693	<0.001
Probability of ejaculation	Treatment*Copulation*Female.order	1	0.724	0.395
	Treatment*Copulation	1	0.046	0.831
	Treatment*Female.order	1	0.037	0.847
	Copulation*Female.order	1	0.183	0.669
	Treatment	1	<0.001	0.976
	Female.order	1	29.845	<0.001
	Copulation	1	31.147	<0.001
Standardised sperm number	Treatment*Copulation*Female.order	1	0.353	0.552
	Treatment*Copulation	1	1.008	0.315
	Treatment*Female.order	1	1.996	0.158

		Copulation*Female.order	1	3.303	0.069
		Treatment	1	0.091	0.763
		Female.order	1	52.408	<0.001
		Copulation	1	32.293	<0.001
Latency to ejaculate		Treatment*Copulation*Female.order	1	0.406	0.524
		Treatment*Copulation	1	0.458	0.498
		Treatment*Female.order	1	11.140	<0.001
		Copulation*Female.order	1	5.303	0.021*
		Treatment	1	0.021	0.886
		Female.order	1	75.545	<0.001
		Copulation	1	49.2	<0.001

* no longer significant after Bonferroni correction (new significance threshold 1.25%)

Aim 2. To test whether male ejaculate expenditure is influenced by uncertainty

(i) *Comparing T1 with T3.1*

When comparing T1 and one-female trials in T3, there was no evidence of treatment effects in any of the four male responses investigated (i.e. probability of mating, probability of ejaculation, standardised sperm number, the latency to ejaculate) after Bonferroni correction (Table 3, Fig. 2a-d).

Table 3. The analysis of variance (ANOVA) table:

glmer or lmer on the probability of mating, probability of ejaculation, standardised sperm number and latency to ejaculate of the training trials of Treatment 1 and 3.1				
Response variable	Factors	df	Chisq	P value
Training trials of Treatment 1 and 3.1				
Probability of mating	Treatment*Copulation*Female.order	1	1.190	0.275
	Treatment*Copulation	1	0.065	0.799
	Treatment*Female.order	1	0.535	0.464
	Copulation*Female.order	1	22.383	<0.001
	Treatment	1	0.228	0.633
	Female.order	1	292.35	<0.001
	Copulation	1	75.35	<0.001
Probability of ejaculation	Treatment*Copulation*Female.order	1	0.985	0.321
	Treatment*Copulation	1	0.949	0.330
	Treatment*Female.order	1	<0.001	0.993
	Copulation*Female.order	1	8.935	0.003
	Treatment	1	0.471	0.492

	Female.order	1	33.964	<0.001
	Copulation	1	22.68	<0.001
Standardised sperm	Treatment*Copulation*Female.order	1	0.029	0.864
	Treatment*Copulation	1	2.154	0.142
	Treatment*Female.order	1	1.856	0.173
	Copulation*Female.order	1	2.237	0.135
	Treatment	1	0.453	0.501
	Female.order	1	49.277	<0.001
	Copulation	1	51.345	<0.001
Latency to ejaculate	Treatment*Copulation*Female.order	1	0.361	0.548
	Treatment*Copulation	1	0.729	0.393
	Treatment*Female.order	1	4.937	0.026*
	Copulation*Female.order	1	5.498	0.019*
	Treatment	1	0.456	0.500
	Female.order	1	89.29	<0.001
	Copulation	1	32.003	<0.001

*no longer significant after Bonferroni correction (new significance threshold 1.25%)

(ii) Comparing T2 with T3.2

When comparing T2 with two-female trials in T3, the probability of mating declined faster for Treatment 2 males than for Treatment 3 males when exposed to two female trials, represented by a weakly significant Treatment by Copulation order effect. However, the effect lost significance after Bonferroni correction (Table 4, Fig.2a). There was no treatment effect in terms of the probability of ejaculation (Table 4, Fig 2b). But for the standardised sperm number, Treatment 2 males invested significantly more sperm in the second half of the trial, represented by a significant Treatment by Female order effect though this effect lost significance after Bonferroni correction (Table 4, Fig 2c). As for the latency to ejaculate, Treatment 2 males' sexual interest declined faster than Treatment 3 two-female males. However, the Treatment by Copulation order effect lost significance after Bonferroni correction (Table 4, Fig 2d).

Table 4. The analysis of variance (ANOVA) table:

glmer or lmer on the probability of mating, probability of ejaculation, standardised sperm number and latency to ejaculate of training trials of Treatment 2 and 3.2				
Response variable	Factors	df	Chisq	P value
Training trials of Treatment 2 and 3.2				
Probability of mating	Treatment*Copulation*Female.order	1	0.604	0.437
	Treatment*Copulation	1	3.904	0.048*
	Treatment*Female.order	1	3.466	0.063
	Copulation*Female.order	1	4.84	0.028
	Treatment	1	0.557	0.456
	Female.order	1	71.972	<0.001
	Copulation	1	140.22	<0.001

Probability of ejaculation	Treatment*Copulation*Female.order	1	1.211	0.271
	Treatment*Copulation	1	0.128	0.721
	Treatment*Female.order	1	0.319	0.572
	Copulation*Female.order	1	1.359	0.244
	Treatment	1	<0.001	0.987
	Female.order	1	37.151	<0.001
	Copulation	1	63.788	<0.001
Standardised sperm count	Treatment*Copulation*Female.order	1	0.034	0.854
	Treatment*Copulation	1	0.526	0.468
	Treatment*Female.order	1	4.712	0.030*
	Copulation*Female.order	1	0.949	0.002
	Treatment	1	0.019	0.891
	Female.order	1	70.391	<0.001
	Copulation	1	45.33	<0.001
Latency to ejaculate	Treatment*Copulation*Female.order	1	0.487	0.485
	Treatment*Copulation	1	5.811	0.016*
	Treatment*Female.order	1	0.125	0.724
	Copulation*Female.order	1	1.238	0.266
	Treatment	1	0.564	0.452
	Female.order	1	70.071	<0.001
	Copulation	1	127.71	<0.001

* no longer significant after Bonferroni correction (new significance threshold 1.25%)

Aim 3. To investigate how past experience influences ejaculate expenditure patterns

When comparing male behaviour at the fourth mating opportunity across an initial stage of the training trials, the rest of the training trials, and the final trial, we found a tendency (no longer significant after Bonferroni correction) for standardised sperm numbers to vary across the three stages, with sperm numbers being lower in the first trial across all treatments, and a non-significant tendency to vary across the three treatments. Post-hoc tests confirmed no significant differences across treatments (Table 5, Fig. 2a). Males were consistently slower to ejaculate in their first training trial than in the last final trial across all treatments (Table 5, Fig. 2b). Males were also consistently slower to ejaculate in T1 than in T2 (Table 5, Fig. 2b).

Table 5. The analysis of variance (ANOVA) table:

glmer or lmer of the standardised sperm number and latency to ejaculate across treatments in three stages				
Response variable	Factors	df	Chisq	P value
Comparison of Trial 1, Trial 2-14, and Final trial across all treatments				
Standardised sperm number	Stage*Treatment	4	1.198	0.878
	Stage	2	6.632	0.036*
	Treatment	2	4.720	0.094
Latency to ejaculate	Stage*Treatment	4	1.352	0.853
	Stage	2	11.07	0.004
	Treatment	2	10.736	0.005

* no longer significant after Bonferroni correction (new significance threshold 1.25%)

Post-hoc

Standardised sperm: no significant pairs

Latency to ejaculate: First trial vs Final trial: $P=0.020$

Treatment 1 vs Treatment 2: $P=0.017$

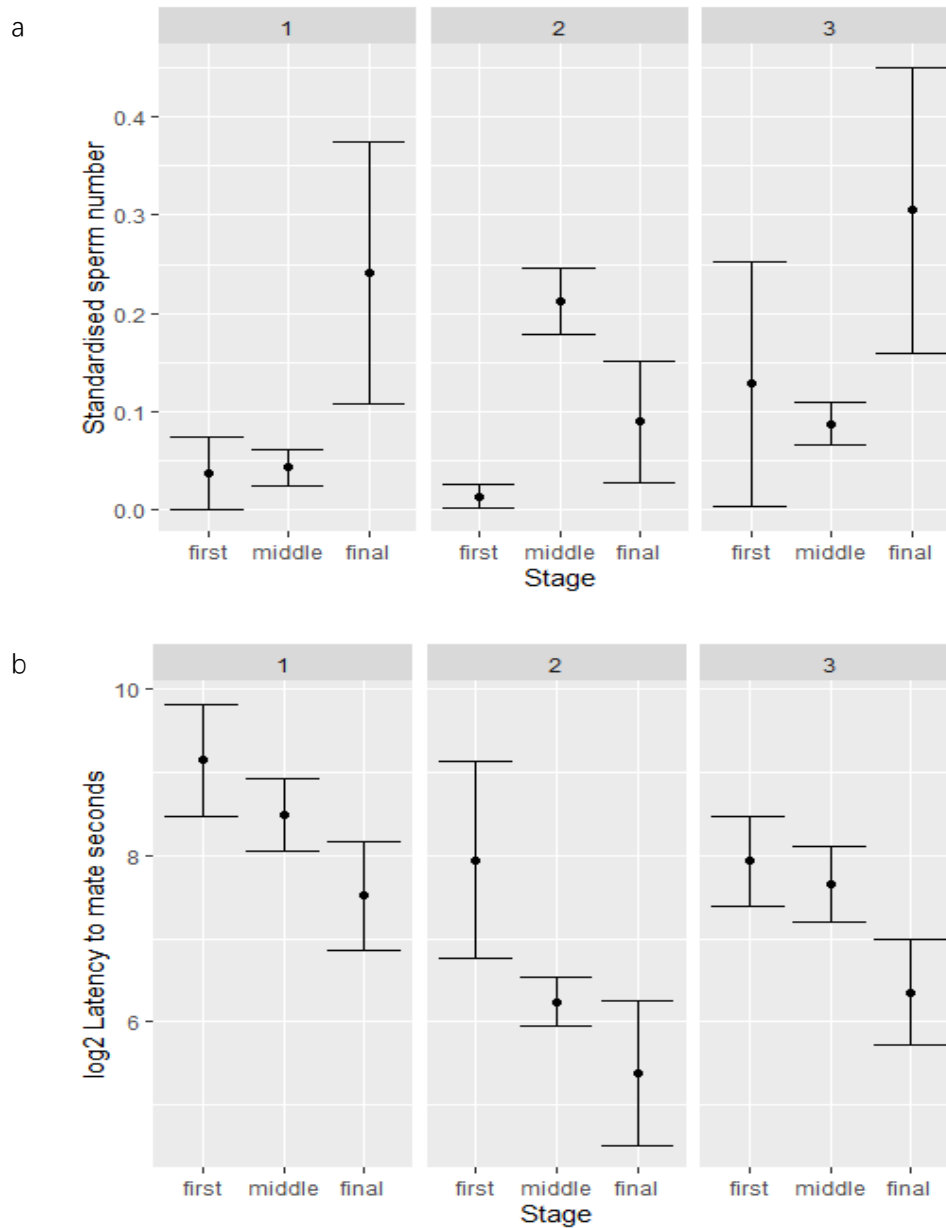


Figure 3. (a) The standardised sperm number and (b) The latency to ejaculate of the fourth copulation over successive copulations in three treatments in the training stage, $\log_2(\text{Time} + 1)$. Those are plots of the means of all males in each treatment. Vertical bars = SE. First trial represents trial 1. Middle trials represent trials 2 to 13 or 14. Final trial represents the last trial, i.e. trial 14 or 15.

Discussion

The aims of this study are (i) to test male preferential expenditure in a sexually novel female controlling for mating order; (ii) to test whether male ejaculate expenditure is influenced by uncertainty; and (iii) to investigate how past experience influences ejaculate expenditure patterns. Here is the summary table of all significant results associated with Treatment or experience.

Comparison	Response variable	Significant terms	P value
T1 vs T2	Probability of mating	Treatment*Copulation	0.004
		Treatment*Female order	0.004
	Probability of ejaculation	Treatment*Copulation	0.025*
	Standardised sperm number	Treatment*Female order	0.026*
T3.1 vs T3.2	Probability of mating	Treatment*Copulation	<0.001
		Treatment	<0.001
	Latency to ejaculate	Treatment*Female order	<0.001
T1 vs T3.1	Latency to ejaculate	Treatment*Female order	0.026*
T2 vs T3.2	Probability of mating	Treatment*Copulation	0.048*
	Standardised sperm number	Treatment*Female order	0.03*
	Latency to ejaculate	Treatment*Copulation	0.016*
Experience ¹	Latency to ejaculate	Stage	0.004
		Treatment	0.005

* no longer significant after Bonferroni correction (new significance threshold 1.25%)

¹ post-hoc tests: P (First trial vs Final trial) = 0.02, P (T1 vs T2) = 0.017

Aim 1. To test male preferential expenditure in a sexually novel female controlling for mating order

We found that males had a significantly higher probability of mating and ejaculated

significantly faster in the second half of a trial in Treatment 2, compared to males in Treatment 1. For Treatment 3, the probability of mating in the second half of a trial was significantly lower in one female sub-treatment than in the two female sub-treatment as reflected by a significant Treatment by Female order interaction and the latency to ejaculate was significantly smaller in two-female sub-treatment. The results suggest the male strategically allocate sperm mainly by strategical mating. These differences suggest that males have a heightened sexual interest with a second female, which supports the Bateman's gradient that males should increase their mate number to achieve the higher reproductive success. For the probability of ejaculation and the standardised sperm, there is no significant Treatment or interaction with Treatment effect. For the sperm count, there is no treatment effect. For the latency to ejaculate, the treatment by copulation interaction term was significant comparing Treatment 1 and Treatment 2, while the treatment by female order interaction was significant comparing Treatment 3.1 and Treatment 3.2. Collectively, the results substantiate the Coolidge effect in red junglefowl using control groups and disentangle the effect of copulation order.

For Treatment 1, we observed a downward exponential curve. This indicates that males invest fewer sperm in subsequent copulations. A short-lived Parasitic wasp, *Trichogramma evanescens*, males have a finite amount of sperm. They transfer a similar amount of sperm for the first ten females for each mating³⁷. On the contrary, the decline in sperm number for red junglefowl was immediately. What is the reason that they did not invest an equal amount of sperm in successive copulations with the same female? My explanation is, that there may be a temporal discounting in sperm allocation. Temporal discounting, the process of systematically devaluating outcomes over time, is a typical process in sequential encounters. A temporally proximal small reward may be preferred over a temporally distant large reward³⁸. Two mechanisms may explain this. The first is the uncertainty of future rewards. Reinhold and Fryer argued that males usually spend more sperm in initial copulations because male mortality risk may

devalue male future reproductive success^{39,40}. Males should prefer to invest more sperm in more certain mating opportunities (current mating opportunities) than more uncertain ones (future mating opportunities). The individual may not be able to live long enough till the larger reward is granted, or the potential larger reward, a mate or a prey, might get lost. Therefore, male preference can be reversed if the mating is delayed. For example, in a dusky salamander, *Desmognathus santeetlah*, males mated with low- and high-quality females at similar rates when females were presented sequentially, but prefer to mate with high-quality females when presented simultaneously⁴¹.

Secondly, even if a larger reward in the future is guaranteed, individuals need self-control to accept delayed gratification. A series of slightly different marshmallow tests were carried out. The key experimental design is if the kids can wait for some time to eat one marshmallow, they can get an additional reward. Those who waited to get more rewards are deemed to have more self-control⁴²⁻⁴⁴. Similar tests were done for animals. If a parrot can wait 15 minutes, it will get two instead of one treats and parrot learned to wait for a better reward⁴⁵. Instant satisfaction weighs more than the same reward in the future. In nature, males mostly encounter females sequentially^{46,47}. Though the temporal discounting and delayed gratification are studied extensively in foraging, little is known about that in ejaculate expenditure. We already know that ejaculation induces a reward state in males⁴⁸ and sexual arousal is an important predictor of sperm concentration⁴⁹. But the exact relationship of the rewarding levels and ejaculate size is unclear yet. Males may receive a larger reward when the ejaculate size is large. Therefore, the Coolidge effect suggests that males resist a small sexual reward now and wait for a larger reward in the future.

For Treatment 2 males might delay sexual gratification to get a larger reward later than get a smaller reward sooner. According to the Bateman paradigm, males benefit from having more mates. Therefore, males were facing a choice at the third copulation, whether to invest sperm in the current familiar female or wait 15 minutes to get a more

favourable novel female. Males invested significantly more sperm in the second female. The cumulative sperm number of six copulations did not differ significantly between treatments (Appendix Table A1, Fig. A1) which indicates males decreased the amount of sperm in the first female but shifted it to the second female, suggesting delayed gratification. Delayed gratification was only studied in terms of foraging in several species, like humans ^{42-44,50}, pigeons and rats ⁵¹ before. However, for Treatment 3 two subgroups, males invested the same amount of sperm in the first three copulations but Treatment 3.2 males invested significantly more in the second half of the trial compared to Treatment 3.1 males, suggesting that rather than adjusting sperm allocation, males allocated more sperm in Treatment 3.2 than in Treatment 3.1.

Aim 2. To test whether male ejaculate expenditure is influenced by uncertainty

Galvani proposed models which predict that when males mate sequentially while sperm stores are limited, they should prefer to withhold sperm when the future mating opportunity is uncertain. For Treatment 1 males, the future mating is certain that they would only have one female per trial. They are predicted to invest more in the first female during the first half of a trial. For one female subgroup Treatment 3 males, the future mating is less certain that they might get an additional female (the same or the better compared to Treatment 1). So they are predicted to reserve some sperm. However, we did not observe any differences between the two treatments. It is possible that the effect size is small and a small sample size is not able to test that.

For Treatment 3.2, males still invested more sperm in the novel female than in the familiar female. However, the magnitude of the difference is smaller than that of treatment 2. The average of standardised sperm number in copulation 4 in Treatment 2 is 0.20, and two females in Treatment 3 is 0.13. This suggests that males can adjust their delayed gratification under uncertainty. Treatment 3.2 are less certain about the number of females they would get (the same or the worse compared to Treatment 2). So they

would preferentially invest in current more certain copulations and reserve less for the potential novel female. Furthermore, the difference within Treatment 3 is approximately a half in the difference between Treatment 1 and 2. This also suggests males have statistical inference on the probability of encountering a second female which is 50%. Animals like kea showed the ability to do statistical inference⁵². Other animals like bonobos reduced delayed gratification under uncertainty as well⁵³. This is the first study to show the statistical inference in the contexts of making sexual decisions.

Aim 3. To investigate how past experience influences ejaculate expenditure patterns

As for the training process, before the experiment, males were kept in single sexed pens and had very scarce mating opportunities. So in the first trial, Treatment 2 males used most sperm in the first three copulations and almost did not reserve sperm. Later, they 'learned' they would always get two females, so they can plan to reserve some sperm for the second female, as indicated by the fact that Treatment 2 males invested more sperm in the fourth copulation in the training stage. However, this Stage effect is not significant, possibly due to the limited number of data points. But for the latency to ejaculate, males waited significantly longer to ejaculate in the first trial than in the final trial. One possible explanation is that in the final trial, they learned they can get a second female and were prepared for that. But in the first trial, their sperm reserves were exhausted after repeated mating with the first female and they were not prepared for a second female. Across all stages, Treatment 1 males waited significantly longer to ejaculate than Treatment 2 males. The results substantiate the Coolidge effect as well.

For the final trial, Treatment 1 and Treatment 3 instead of Treatment 2 males invest more sperm in the second female though this effect is not significant. As for Treatment 1 and Treatment 3 males, they might have learned that female availability was high even if they only had one female per trial, compared to situations when they were kept

in single-sex pens. When the female availability is high, males are more prudent and they tend to reserve some sperm in case they meet a novel female or a higher-quality female in the future. Though the standardised sperm number of the 4th copulation does not show any significant effects, when we compared standardised sperm number across 6 copulations through all three treatments, we found a significant difference (Appendix Table A2, Fig. A2). However, the patterns are not consistent with any predictions.

But it is not clear why Treatment 2 males invest fewer sperm in the final trial than in the training trials. The reasons could either be that the sample size is small so the results might not be representative or they might get habituated to the paradigm and stopped actively reserving sperm for the second female.

Conclusions

The study provides an experimental demonstration that male red junglefowl invests preferentially in sexually novel females, substantiating previous work. This bias is largely driven by decisions to mate and ejaculate rather than differences in sperm numbers. The study also suggests that the uncertainty of encountering a second female can reduce preferential investment in female novelty. Finally, we found some weak evidence suggesting that previous experience may influence current ejaculate expenditure behaviour although these patterns warrant further investigation.

Appendix

Cumulative sperm number

We fitted a generalised linear mixed effect model to test if the cumulative sperm number of six copulations change over time and if there is a difference across treatments. The response variable is cumulative sperm number, the fixed effect is treatment by trial, male ID and female ID as random effects. Then, we used a chi-squared test to test whether there is a significant effect. The cumulative sperm number decreases over successive trials in Treatment 1 but increases in Treatment 2 and 3 (Fig. A1). No significant difference is detected (Table A1).

Table A1. Cumulative sperm number

Response variable		Factors	df	Chisq	P value
Training trials					
Cumulative sperm number	sperm	Treatment*Trial	2	3.0397	0.2187
		Treatment	2	0.0588	0.971
		Trial	1	0.3242	0.5691

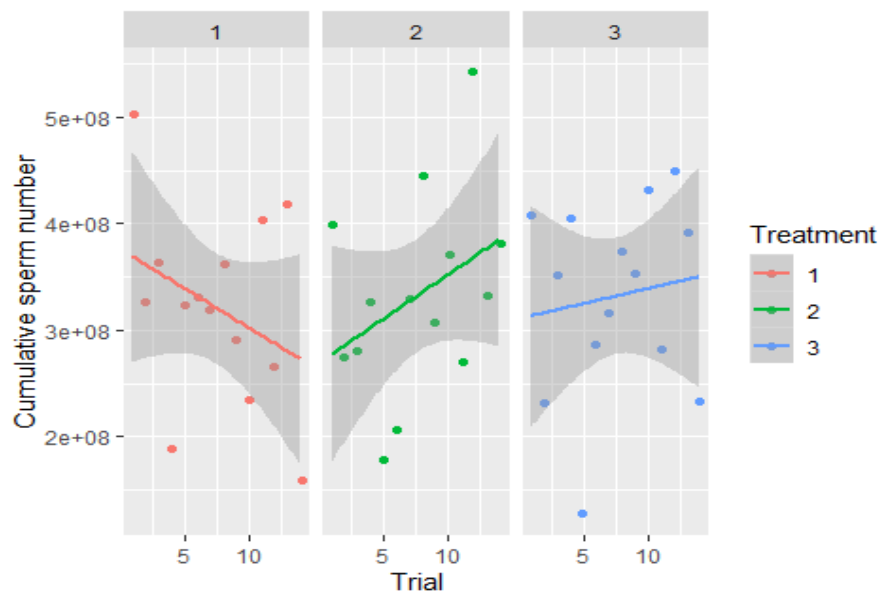


Figure A1. Cumulative sperm number over successive trials. In Treatment 1,

cumulative sperm number decreases over trials while in Treatment 2 and 3, cumulative sperm number increases over trials.

Final trials across treatments

We conducted the same statistical analyses as that in Table 1-4 in the main text. We fitted three generalised linear mixed effect models, with the probability of mating, probability of ejaculation, and the standardised sperm number, and one linear mixed effect model with log2 (time+1) as the response variables, with Treatment*Female order*Copulation order as fixed effects, male ID and female ID as random effects. Afterwards, we used a chi-squared test to test whether the treatments or the interaction with treatment are significant across three treatments. The probability of mating in the second half of the trial differed significantly across treatments (Table 2, Fig. 2).

Table 2. Final trials across treatments

Response variable	Factors	df	Chisq	P value
Final trials of three treatments				
Probability of mating	Treatment*Copulation*Female.order	2	2.746	0.253
	Treatment*Copulation	2	0	1
	Treatment*Female.order	2	25.084	<0.001
	Copulation*Female.order	1	0	1
	Treatment	2	0.402	0.818
	Female.order	1	21.772	<0.001
	Copulation	1	49.117	<0.001
Probability of ejaculation	Treatment*Copulation*Female.order	2	5.967	0.051
	Treatment*Copulation	2	0.792	0.673
	Treatment*Female.order	2	0.944	0.624
	Copulation*Female.order	1	0.368	0.544
	Treatment	2	0.285	0.867

		Female.order	1	2.781	0.095
		Copulation	1	3.269	0.071
Standardised sperm number		Treatment*Copulation*Female.order	2	2.077	0.354
		Treatment*Copulation	2	2.541	0.281
		Treatment*Female.order	2	0.291	0.865
		Copulation*Female.order	1	3.762	0.052
		Treatment	2	0.538	0.764
		Female.order	1	8.730	0.003
		Copulation	1	9.625	0.002
Latency to ejaculate	to	Treatment*Copulation*Female.order	2	0.509	0.775
		Treatment*Copulation	2	7.159	0.028*
		Treatment*Female.order	2	1.916	0.384
		Copulation*Female.order	1	1.565	0.211
		Treatment	2	10.251	0.006
		Female.order	1	16.688	<0.001
		Copulation	1	16.195	<0.001

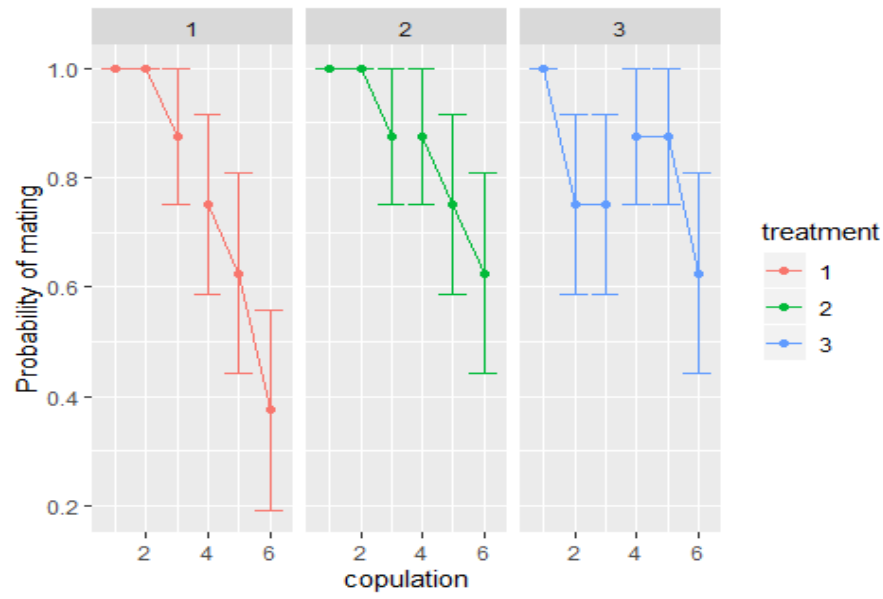
Post-hoc

Mating T1 vs T2 P= **<.0001**, T1 vs T3 P= **<.0001**, T2 vs T3 P= **<.0001**

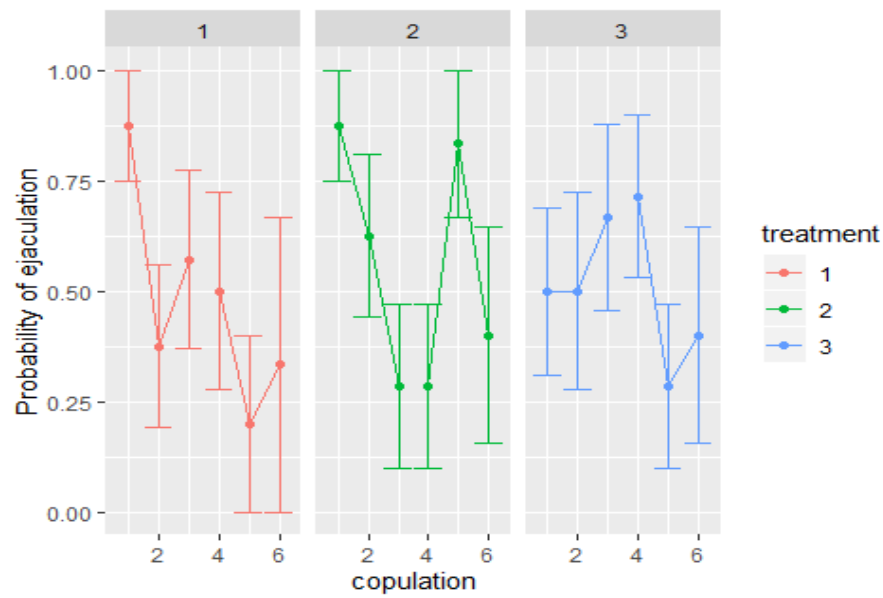
Time T1 vs T2 P= **0.004**

*no longer significant after Bonferroni correction

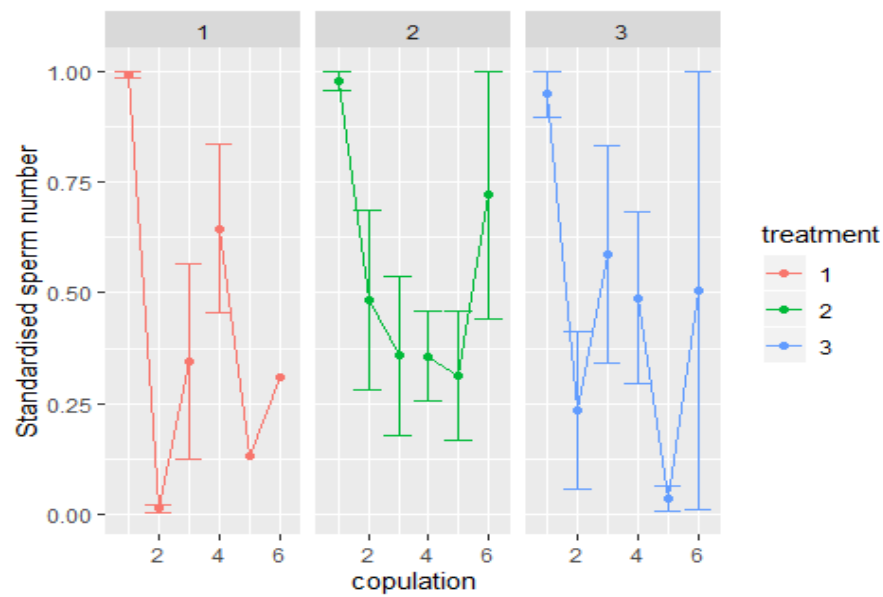
a



b



a



b

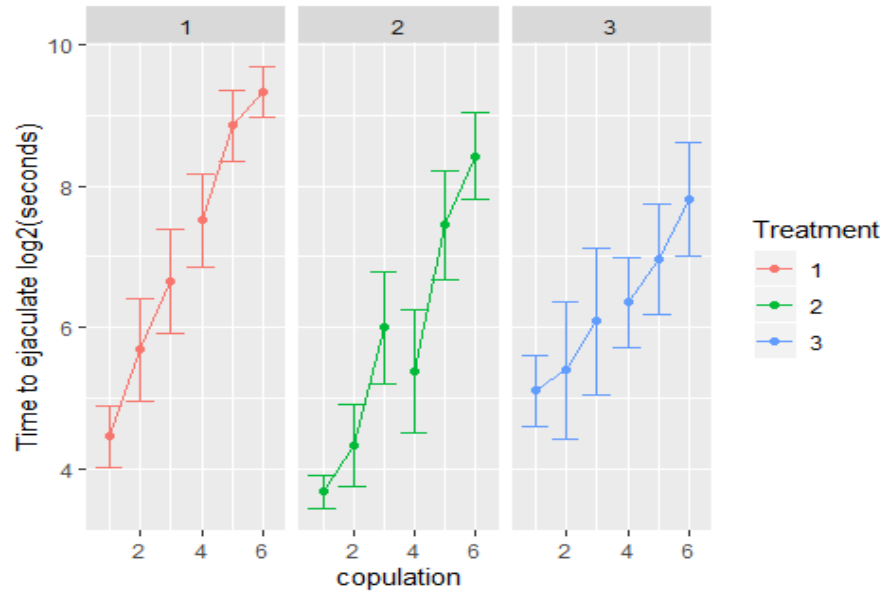


Figure 2. (a) Probability of mating, (b) probability of ejaculation, (c) standardised sperm numbers, and (d) latency to ejaculate ($\log_2(\text{Time} + 1)$) over successive mating opportunities in treatment 1 (1), treatment 2 (2), and treatment 3 in the final trial. Data points represent means of all males in each treatment. Vertical bars = SE.

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Chapter 6. Discussion

Overview

Optimising ejaculate expenditure within physiological and cognitive constraints represent a critical component of male reproductive fitness, particularly in polygynandrous species, where mating with multiple females can severely deplete male sperm reserves and sperm competition favours investment in large, high-quality ejaculates. Despite increasing interest in ejaculate expenditure strategies however, little remains known about the different mechanisms that can modulate or constrain male investment. The overarching goal of this project was to investigate patterns of male investment in mating opportunities and expenditure in natural ejaculates in the red junglefowl, *Gallus gallus*, a seasonal polygynandrous bird. I explored four aspects: (1) seasonal and circadian patterns of ejaculate expenditure; (2) the dynamic relationship between male social status and ejaculate expenditure; (3) the role of past experience male ejaculate expenditure according to female reproductive quality; (4) male ejaculate expenditure in with respect to female sexual novelty and the role of male expectations of female availability.

Chapter 2 experimentally investigated the extent to which different measures of male investment in a mating opportunity changed over the course of the breeding season and at different times of day. We investigated seasonal patterns by combining data obtained from three different experiments, which followed a similar protocol. We found that males in this study population, gradually increased their sperm investment since April and sperm allocation peaked in June and decreased later in the season. We investigated circadian patterns with an additional experiment where a set of males was tested in the morning, midday and afternoon. We found that contrary to previous studies in domestic fowl, males were insensitive to the time of the day in terms of sperm allocation and latency to ejaculate.

Chapter 3 aimed to test for a relationship between male status and ejaculate expenditure as well as its causality. We experimentally assembled pairs of males and monitored their social hierarchy. We collected one ejaculate from both the dominant and the subordinate member of each pair. We subsequently reshuffled pairs, matching two dominant and two subordinate males together, which forced one male in each new pair to change status. We collected one ejaculate from each male again. Contrary to previous work, we found that male social status was not a significant predictor of male ejaculate expenditure. Furthermore, we did not find a significant change in ejaculate expenditure resulting from a change in a male's social status.

In chapter 4, we conducted an experiment to test whether males prefer large-combed females in sequential mating, and how the experience of female quality in the past influences current ejaculate expenditure. Each male consistently received either a small-combed or a large-combed female in each of four trials in the training phase and was allowed to mate with the female up to 6 times. In a final fifth trial, both males that had been exposed to large-combed or small-combed females, were exposed to the same female of intermediate comb size. We found that in the first 4 trials, males exposed to large-combed females had consistently higher ejaculate expenditure than males exposed to small-combed females, suggesting that males have the ability to assess female reproductive quality, and male choice operates mainly cryptically (i.e. through differential sperm investment). Importantly, we also found that a male's ejaculate expenditure in the intermediate-combed female in the final trial was similar to his expenditure in the previous four trials. Thus, the intermediate-combed female received more sperm from males that had previously encountered large- versus small-combed females. These results suggest that males may have a carry-over effect where consistent previous experience influences ejaculate expenditure in the current mating opportunity.

Chapter 5 followed up previous work indicating male preferential investment in sexually novel females in this species, and experimentally tested whether increased

male ejaculate expenditure is caused by female novelty, and whether previous patterns of female availability enable males to anticipate and respond to exposure of a novel female. We exposed each male to 13-14 training trials from one of three treatments. In treatment 1 (T1) males received one female per trial and could mate up to 6 times, in treatment 2 (T2) males received the first female was replaced by a novel second female after the 3rd mating opportunity, and in treatment 3 (T3) males were exposed to trials identical to T1 (6 mating opportunities with one female) or T2 (3 mating opportunities with each of 2 females presented sequentially) in random order but with equal probability. Following these training trials, all males across the three treatments were exposed to a final trial identical to T2, i.e. 3 mating opportunities with each of 2 females presented sequentially. We found that ejaculate expenditure declines over successive mating of a trial, consistent with sperm depletion. However, expenditure in the last 3 mating opportunities was higher in T2, where males were exposed to a novel female, compare to T1 where the female was familiar. T3-males displayed an overall intermediate response, and the effect of female novelty was further demonstrated when comparing male expenditure in T3 trials with two females versus T3 trials with a single female. Thus, these results confirm that the previously described male preference in sexually novel females (Coolidge effect) is caused by female novelty rather than by mating order effects. We also found that in the first trial, when males were naïve with respect to experimental treatments, expenditure patterns were similar across treatments and showed no evidence for a preferential investment in novel females, suggesting that males may require previous experience to generate an expectation of the novelty of the female in the next mating opportunity. There was also some evidence that males exposed to different treatments (T1-T3) had different patterns of ejaculate expenditure in the final trial. However, the extent to which these differences were explained by previous experience is less clear.

Significance of our findings

Kelly and Jennions (2011) reviewed that a series of theoretical models predict how

ejaculate size will be strategically adjusted in relation to the number of immediate rival males, female mating status (though results are less clear), and female quality (i.e. female size or condition). Males were found to be able to adjust rapidly in ejaculate size depending on the relevant social context which is the strategic sperm allocation ¹. Males invest significantly more sperm in high quality females and when the number of rivals increased from 0 to 1 (risk of sperm competition). Bretman et al. (2011) also reviewed that fully plastic behaviours are widespread and are selected in highly variable environments ². Mery and Burns (2010) proposed that animal plasticity either generated from an evolutionary pre-programmed cue-response behaviour or learning from experience³. Collectively, this work made a number of contributions and bridged the gap between plastic sperm investment and their cognitive mechanism.

By collecting and analysing temporal variation in natural ejaculates, these results fill the gap between information on seasonal patterns of spermatogenesis and seasonal patterns in ejaculate expenditure behaviour. These findings indicate that previous reports of negative relationships between ejaculate traits and social status in domestic fowl are not confirmed in this population of red junglefowl, contributing to the study of alternative mating tactics in polyandrous species. Our results shed novel light on mechanisms of male mate choice by showing that males might use different context-dependent mechanisms of mate choice when exposed to sequential mating opportunities. Importantly, we show novel evidence of strong carry-over effects in male ejaculate expenditure responses to female quality, and demonstrate that male preferential expenditure toward novel females is influenced by patterns of female availability.

Key considerations emerging across chapters

A number of questions emerge from this collective body of empirical work. Here we discuss the most relevant implications of the key aspects of these findings. The discussion is centred on five main considerations or questions.

- (i) The findings of chapters 4 and 5 suggest that males curtailed their ejaculate expenditure and did not use up their entire extragonadal sperm reserves when consistently exposed to small- and intermediate-combed females. While this response is intuitive when there is a prospect to encounter a higher-quality female in the near future, it is more difficult to understand when the experimental design removes this possibility, why did then males curtail their ejaculate expenditure?
- (ii) Our results of chapters 4 and 5 also indicate that males invested overall more sperm in one large-combed female than in two intermediate-combed females. Again, the functional significance of this response is unclear. Is that adaptive or maladaptive?
- (iii) Males display a preference for sexually novel females largely in terms of whether they mate or not with a female, and a preference for high-quality (large-combed) females mainly cryptically, through the number of sperm allocated. Why do mechanisms of male choice in the context of novelty vs familiarity differ from those underpinning mate choice in the context of female reproductive quality?
- (iv) Previous experience is predicted to influence current ejaculate expenditure strategies. However, across chapters 3-5, the way in which past experience appears to influence male decision-making is not consistent.
- (v) Finally, the findings of chapter 2 and 3 were inconsistent from those of similar previous studies.

- (i) Why did males appear to curtail ejaculate expenditure when they are unlikely to encounter new females in the near future?

The Coolidge experiment in chapter 5 and the female quality experiment in chapter 4 followed a similar protocol. In chapter 5 females were of intermediate quality (comb size) while in the first four trials in chapter 4 females were of either high- or low- quality. If we compared the average cumulative number of sperm invested by a male in intermediate-quality females in the chapter 5 experiment (focusing on treatment where only one female was presented in a trial) with the cumulative number of sperm invested

in either high- or low- quality females in chapter 4, we find that males invested most in high-quality females (507.1 ± 46 million, $n=9$, chapter 4), then in the intermediate-quality females (327.2 ± 87.2 million, $n=8$, Treatment 1 males, chapter 5) and least in the low-quality females (228.8 ± 58.4 million, $n=10$, chapter 4). This pattern is intuitive when male expenditure in a female is traded-off against expenditure in future females. However, this was not the case in our experiments. Sperm are continuously produced in male red junglefowl, and accumulate in the vas deferens as extragonadal sperm reserves. Ejaculate expenditure draws on extragonadal sperm reserves. Males can fully replenish their extragonadal sperm reserves within 48 hours⁴. The time between trials was 48 hours specifically to ensure that males would start each trial with fully replenished sperm reserves. It seems that males reserve sperm even when there is no foreseeable mating opportunity within 48 hours. It is counterintuitive that they prefer to reserve sperm which would subsequently be lost than to invest more in intermediate- or low-quality females. If males learned that no additional female would become available for the next 48 hours, one would expect them to invest maximally in the current female regardless of her reproductive quality.

Several mechanisms could explain this. Firstly, this response may be maladaptive. For example, it is possible that males did not learn the experimental pattern. Males may either not be able to learn this pattern or they may require longer to learn. In this case, males may have operated under the assumption that they might encounter new females in the near future (i.e. less than 48 hours from the current trial). Secondly, while unable to learn the exact pattern of female provision, males may instead respond to their temporary isolation from other male competitors. Absence of competitors may translate into higher availability of females, and this would promote economical ejaculate expenditure. So because of isolation from rivals, males may have been more inclined to reserve sperm for an unexpected mating. A third scenario is that this might represent an adaptive response. Males learned but still chose to reserve some sperm for unexpected events. They were only exposed to 4-14 training trials depending on

different experiments. Even if males learned the pattern, they could not rule out the possibility of encountering a new female before the next 48 hours. Fowl social units are highly fluid and males may be selected to budget for some uncertainty in their ejaculate expenditure decisions⁵. It is beneficial to reserve some sperm under an uncertain world. Furthermore, low-quality females attract fewer males and males may experience a lower sperm competition risk when mating with these females compared to when mating with high-quality females which are likely associated with intense sperm competition. Theory predicts that when there is an elevated risk of sperm competition, males should invest more sperm⁶. But when there is elevated intensity of sperm competition, males should decrease sperm investment as the number of competing ejaculates exceeds two⁷. This is because there are diminishing returns for providing more sperm. Empirical evidence was found as well⁸⁻¹⁰. Thus, under sperm competition risk theory, males are predicted to invest more sperm in high-quality females. Under sperm competition intensity theory, males are predicted to invest less in high-quality females when the sperm competition intensity is high. Since intermediate- and low-quality females potentially mate with fewer males and incur a lesser sperm competition. The marginal gain to mate with low-quality and intermediate-quality females may not be large enough to offset the benefits of reserving sperm. Reserving sperm for the future may potentially yield more return.

(ii) Why did males invest more sperm in one large-combed female than two intermediate-combed females?

On average males invested fewer sperm in 6 matings with two sequentially presented intermediate-quality females (treatment 2 of the Coolidge experiment in chapter 5, 331.2 ± 31 in millions, $n=8$) than in six matings with a single high-quality female (large-combed female treatment of the female quality experiment in chapter 4, 507.1 ± 46 , $n=19$). Specifically, males invested 481 ± 48.1 million sperm in the first three copulations and 26.4 ± 7.89 million sperm in the last three copulations for high-quality

females. Males invested 275.4 ± 30 million sperm in the first three copulations and 55.8 ± 9.81 million sperm in the last three copulations for intermediate-quality females.

A possible explanation is that it may be more beneficial to invest sperm in one high-quality female than in two intermediate-quality females. This may happen for example, if female reproductive quality scales non-linearly with comb size and the reproductive returns of mating with a large-combed female greatly exceed the benefits of mating with an intermediate-combed female. In addition, the optimal strategy might not always be to invest sperm in as many females as possible during a single reproductive cycle. The number of offspring that a male can expect to sire with a mate may progressively decline due to limits in the ability to provide paternal care or nuptial gift ¹¹. A theoretical model postulates that the optimal strategy for a lone male rat with 4 ejaculates is to deliver two ejaculates to each of two females instead of delivering one ejaculate to four females to ensure fertilization ¹². In marmots, *Marmota flaviventris*, males maintain harems of females and the average number of offspring per female declined with increasing harem size¹³. To reduce the levels of sperm competition, males are selected to guard their mates. However, the guarding durations increase with decreasing female availability and an increasing number of male competitors ¹⁴. Sparing effort to guard one high-quality female may yield more return than guarding two intermediate-quality females. Furthermore, it is predicted that males should invest less when there is no sperm competition. A coarse estimate of essential sperm investment to ensure fertilisation when there is no sperm competition is 50 million sperm. A study found that during artificial insemination when there is no sperm competition, 50 million sperm is enough to fertilise female domestic fowl ¹⁵. The average sperm investment by male junglefowl observed across the experiments of chapter 4 and 5 is beyond this threshold. When female quality is high, the sperm competition will be much higher ^{16 17,18} and males need to allocate more sperm. However, for intermediate-quality females, the risk of sperm competition is low and males may benefit by reducing their investment.

(iii) Why was male choice exerted mainly cryptically in chapter 4 (female

quality experiment), and through mating decisions in chapter 5 (female novelty experiment)?

One possibility is that in chapter 4, it is adaptive to invest fewer sperm but mate equally frequently with low-quality females. In chapter 5, it is adaptive to invest more sperm and mate more frequently with novel females.

Mating and ejaculating serve slightly different functions. Mating *per se* may benefit males independently of the sperm inseminated. In some species, mating can have a multitude of additional functions which include stimulating females to ovulate¹⁹, serve for mate guarding purposes²⁰, inhibit females from remating soon²¹, facilitate the formation of a pair bond²², remove the sperm or copulation plug in the female reproductive tract left by previous males²³. These functions may explain why in chapter 4, males had a similar probability of mating with large or small-combed females but inseminated significantly fewer sperm in small-combed females.

But in chapter 5, males have already mated with the familiar female several times. They did not benefit much from additional mating with or investing additional sperm in the familiar female. Therefore, they mated less frequently in the second half of the trial with the familiar female. When they had a new female, males need to mate at least once with the female to transfer sperm. Therefore, comparing with the familiar female, males mated more often with the novel female. But in terms of the sperm number contained in each ejaculate, males did not invest more sperm in the novel female. This may result from a constraint imposed by depleted sperm reserve.

(iv) Why did the past experience influence current decision-making in different ways across chapters?

The experimental designs for the three chapters are different. Firstly, chapter 3 only has

one training trial. Chapter 4 has four training trials. Chapter 5 has 14 training trials. Since we don't know whether they learned or after how many trials males will eventually learn the pattern. It is possible that for some chapters, training trials are too few for the males to learn. Thus we did not observe an effect. Secondly, chapter 3 focuses on the male side, investigating how male social status influences male mating decisions while chapter 4 and 5 focus on the female side, investigating how the experience of different female quality and female availability influences male mating decisions. Thirdly, chapter 2 only undergoes one copulation per trial while chapter 4 and 5 have up to six copulations in a trial. Male red junglefowl is able to mate multiple times in a short period. There might be a maximal sperm allocation in a single time, which causes the first copulation sperm investment to reach the maximal limit and is not as informative as the cumulative sperm investment which reflects the male's willingness to invest sperm.

Chapter 3-5 have two phases. The first phase is the training phase, and the second is the final experimental phase. How experience influences current sexual decisions can be divided into four categories.

First, no effect is detected. Not all experience is important as a cue for current decision making. For chapter 3, the first trial examines how social status influences sperm allocation strategy. The second trial examines how previous social status influences the current sperm allocation strategy. In the training phase, we found that social status does not affect sexual decisions but males do prefer better quality and novel females. In the final phase, it is reasonable that a non-relevant factor in the past does not affect sperm allocation now. We found that previous social status does not influence current decision making in chapter 3.

Secondly, individuals would behave the same as what they've done before. Males can save energy to avoid making decisions every time. In chapter 4, the training phase

examines male preference for females of different qualities. The final phase examines how previous sexual experience of various female quality influences current sperm allocation strategies in an intermediate-quality female. Males sampled the quality of females in this patch during the training trials and carried over the same strategy in the final trial. In the experiment, the environment is quite stable and it is adaptive for males to turn on 'autopilot' mode. Thus, past experience had an over-riding effect. For example, female guppies, *Poecilia reticulata*, raised with a colourful male preferred colourful males and females raised with non-colourful males preferred non-colourful males²⁴.

Thirdly, individuals would compare current mating opportunities with previous mating opportunities, and adjust sperm allocation. For female choice, experience with an attractive male 24 h earlier caused female field crickets, *Teleogryllus oceanicus*, to mount subsequent males more slowly and retain their spermatophore for less time, whereas experience with an unattractive male caused females to mount subsequent males faster and retain their spermatophores for longer²⁵.

Fourth, circumstances other than the three. For chapter 5, the training phase confirms male preference for novel females. The final phase examines how previous experience of female availability influences current decision making. Experience of the different regimes of female availability appears to contribute to differences in ejaculate expenditure patterns across training trial treatments. The influence of past experience (training trials) on male ejaculate expenditure in the final trial however was less clear.

(v) Why is there inconsistency between some of our results and previous studies?

While this doctoral work was not an exact replicate of previous work, some of our results are inconsistent with the findings of previous studies. In chapter 2, we did not find a significant circadian pattern in the ejaculate expenditure of male red junglefowl

though previous studies found this in domestic fowl²⁶. This might be due to the fact that we only collected one ejaculate from each male in a trial. A single ejaculate might not be large enough to reveal constraints (see discussion in chapter 2). In chapter 3, we did not find a significant social status effect on ejaculate expenditure patterns, though some previous studies found a negative relationship between some measures of ejaculate investment and social status in some populations of domestic fowl^{26,27}. Predictions of the relationship between status and ejaculate quality are however ambiguous. On the one hand, theory predicts that subordinate males should have higher ejaculate expenditure to compensate for increased sperm competition risk and because they invest less in pre-copulatory competition. This scenario assumes that status-specific differences represent adaptive alternative strategies. On the other, differences may reflect differential costs. For example, subordinates may have less resources to allocate to ejaculates or their investment may be inhibited by dominant males.

A more general source of inconsistencies may be low statistical power. Behavioural studies rely heavily on statistics. Though we aim to adopt strict analysis, there is still a small chance of getting false-positive results due to the nature of the way how we reject the null hypothesis. When the effect size is small, we need a larger sample size to achieve reliable power. In chapter 3, we only had a power of 0.8 to detect an effect size that is larger than 0.585. in chapter 2 circadian pattern, we only had a power of 0.8 to detect an effect size that is larger than 0.480. Cohen suggests that 0.1, 0.25, and 0.4 represent small, medium, and large effect sizes respectively. But the differences between different social status or treatments might not be big enough. If we keep the Cohen effect size as 0.25, we got a power= 0.285 for chapter 2, and power= 0.192 for chapter 3.

Future directions and conclusive remarks

The results of this work identify a number of avenues for future research. Firstly, we need to understand the topology of the male reproductive tract and the mechanical and

physiological mechanisms of sperm reservoir and ejaculation. This can give us information about physiological constraints. Secondly, we need to explore the cognitive and neuronal mechanisms of male choice. This could help explain why males sometimes appear to make seemingly maladaptive decisions. Thirdly, there is a need to better understand behavioural plasticity.

To conclude, my thesis has shown that male red junglefowl has plastic patterns of ejaculate expenditure, which are modulated by a number of factors such as seasonality, past experience, female reproductive quality and sexual novelty.

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