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Léna Meunier

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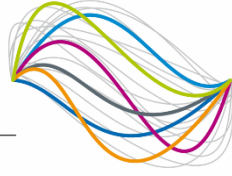
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International Fund For Houbara Conservation



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Doctorat de : Biologie des populations et écologie

Par

Meunier Léna

**Reproductive biology and senescence impact on postcopulatory
sexual selection in a wild polyandrous bird, the North African
Houbara Bustard (*Chlamydotis undulata undulata*)**



Thèse présentée et soutenue à Dijon, le 26/06/2024

Composition du jury:

M. Thierry Rigaud	Directeur de recherche CNRS	Président
Mme. Partyka Agnieszka	Professor, Wrocław University of Environmental and Life Sciences	Rapporteur
M. Rodrigues Silva Alexandre	Professor, Federal University of Semiarid Region	Rapporteur
M. Santiago Moreno Julian	Professor, INIA-CSIC	Examineur
M. Sorci Gabriele	Directeur de recherche CNRS	Directeur de thèse
Mme. Torres Carreira Janaina	Researcher, Reneco International Wildlife Consultants LLC	Co-directeur de thèse

Reproductive biology and senescence impact on postcopulatory sexual selection in a wild polyandrous bird, the North African Houbara Bustard (*Chlamydotis undulata undulata*)

Key words: Sperm competition, reproductive senescence, sperm quality, sperm storage, anatomy, houbara bustard

Abstract:

Polyandry, when females mate with multiple males, increases the opportunity for sexual selection to operate, because in addition to competition between males for access to the female (pre-copulatory sexual selection), sperm from different males also compete for fertilization (post-copulatory sexual selection). This often results in most offspring being sired by the last male, a phenomenon called last male precedence (LMP). In addition to mating order, sperm quantity and quality can also influence the outcome of sperm competition. As males age, sperm quality and quantity decrease, resulting in reduced reproductive success. In the North African Houbara Bustard (*Chlamydotis undulata undulata*), females are polyandrous, and males suffer from reproductive senescence. The thesis aims at improving our understanding of the reproductive biology of houbara bustards, reproductive senescence and its impact on male reproductive success and post-copulatory selection.

In the first part, to improve the knowledge of the species' physiology of reproduction and to construct a solid base for houbara reproductive anatomy and sperm morphology and later storage mechanism in the female tract, we explored anatomy and histology of the female and male reproductive systems during and out of the breeding season and sperm morphology was described. In the second part, we investigated the underlying mechanisms of LMP and the storage dynamics within the oviduct during competitive inseminations. In the third part, we investigated male reproductive senescence and its relationship with post-copulatory selection. This work was divided into four sections to investigate different aspects of reproductive ageing. Firstly, we examined seasonal testosterone levels and a possible age-related pattern. Second, we investigated a possible trade-off between sperm number and quality across ages. Thirdly, to test male age, sperm age, and their possible interaction on sperm quality and reproductive success, sperm ageing in the male tract was investigated by controlling the timing of collection intervals for young and old males. Finally, LMP was studied under controlled experimental conditions to disentangle the effects of male age, mating order, and sperm quality on siring success.

This thesis presents the first description of houbara reproductive anatomy and histology and sperm morphology classification. Active sperm displacement is likely to be the main mechanism of LMP. There was no evidence of an age-related pattern of testosterone levels throughout the breeding season. Peak testosterone levels preceded peaks in sexual behaviour and sperm characteristics. We found a positive covariation between sperm quantity and several sperm quality traits, and male age did not influence the strength of this covariation. No evidence of sperm ageing during prolonged storage in the male tract was observed. On the contrary, sperm collected at longer intervals had higher quality, which was reflected in higher hatching success. Consistent with previous work, older males had lower sperm number, volume, motility and velocity and sired offspring with impaired growth rate. Finally, we found no evidence of an age effect on LMP. Regardless of age, sperm from the last male in the insemination sequence sired approximately 60% of the offspring. Overall, this work has provided a better understanding and new insights into the reproductive biology of houbara bustards and the mechanisms of post-copulatory selection.

Biologie de la reproduction et impact de la sénescence sur la sélection sexuelle post-copulatoire chez un oiseau polyandre sauvage, l'outarde Houbara d'Afrique du Nord (*Chlamydotis undulata undulata*)

Mots-clés: Compétition spermatique, sénescence reproductive, qualité spermatique, stockage spermatique, anatomie, outarde houbara

Résumé

La polyandrie, lorsque les femelles s'accouplent avec plusieurs mâles, augmente les possibilités de sélection sexuelle, car outre la compétition entre les mâles pour l'accès à la femelle (sélection sexuelle pré-copulatoire), les spermatozoïdes des différents mâles sont également en compétition pour la fécondation (sélection sexuelle post-copulatoire). En conséquence, la plupart des descendants sont engendrés par le dernier mâle qui s'est accouplé avec la femelle, un phénomène appelé "last male precedence" (LMP). En plus de l'ordre d'accouplement, la quantité et la qualité des spermatozoïdes peuvent également influencer le résultat de la compétition spermatique, mais avec l'âge, ces paramètres spermatiques diminuent, ce qui réduit le succès reproducteur. Chez l'outarde Houbara d'Afrique du Nord (*Chlamydotis undulata undulata*), les femelles sont polyandres et les mâles souffrent de sénescence reproductive. Cette thèse vise à approfondir notre compréhension de la biologie de la reproduction de l'outarde houbara, de la sénescence reproductive et de son impact sur le succès reproductif des mâles et la sélection post-copulatoire.

Dans une première partie, afin d'améliorer les connaissances sur la physiologie de la reproduction de l'espèce et de construire une base solide pour l'anatomie reproductive et la morphologie des spermatozoïdes, et les mécanismes de stockage chez la femelle, l'anatomie et l'histologie des systèmes reproducteurs femelle et mâle pendant et en dehors de la saison de reproduction ont été explorées et la morphologie des spermatozoïdes décrite. Dans une deuxième partie, les mécanismes sous-jacents la LMP et la dynamique de stockage dans l'oviducte ont été étudiés. Dans une troisième partie, la sénescence reproductive des mâles et sa relation avec la sélection post-copulatoire a été étudiée. Cette partie a été divisé en quatre sections afin d'étudier différents aspects de la sénescence reproductive. Tout d'abord, les niveaux de testostérone au cours de la saison ont été évalués ainsi qu'un éventuel effet de l'âge. Deuxièmement, un potentiel compromis entre le nombre et la qualité des spermatozoïdes en fonction de l'âge du mâle a été exploré. Troisièmement, l'effet de l'âge du mâle, l'âge des spermatozoïdes et leur éventuelle interaction sur la qualité spermatique et le succès reproducteur, ont été étudiés. Enfin, la LMP a été étudiée dans des conditions expérimentales contrôlées afin de démêler les effets de l'âge du mâle, de l'ordre d'accouplement et de la qualité du sperme sur le succès de paternité.

L'anatomie et histologie reproductive ainsi qu'une classification de la morphologie des spermatozoïdes a été décrite pour la première fois chez l'outarde houbara. Le déplacement actif des spermatozoïdes est probablement le principal mécanisme de la LMP. L'âge n'a pas d'effet sur la concentration de testostérone au cours de la saison de reproduction. Les pics de testostérone ont précédé les pics de comportement sexuel et de qualité spermatique. Des covariations phénotypiques positives entre la quantité et la qualité des spermatozoïdes ont été mises en évidence et ne sont pas influencées par l'âge. Aucun signe de sénescence des spermatozoïdes au cours d'un stockage prolongé dans le tractus mâle n'a été observé. Au contraire, les spermatozoïdes collectés à des intervalles plus longs étaient de meilleure qualité, ce qui s'est traduit par un succès d'éclosion plus élevé. Conformément aux travaux antérieurs, l'âge du mâle a impacté la quantité et la qualité des spermatozoïdes, ce qui s'est reflété par un taux de croissance et de survie plus faible sur la progéniture. Enfin, l'âge n'a pas impacté la LMP, et indépendamment de l'âge, le mâle en dernière position dans la séquence d'insémination a engendré environ 60 % de la progéniture. Dans l'ensemble, ce travail de thèse a permis de mieux comprendre la biologie de la reproduction des outardes houbara et les mécanismes de sélection post-copulatoire.

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List of abbreviations

AGEs advanced glycation end products	IPVL inner perivitelline layer
AMPK AMP-activated protein kinase	LH luteinizing hormone
AR acrosome reaction	LM longitudinal manchette
ATP Adenosine triphosphate	LMP Last male precedence
CFC cryptic female's choice	ME median eminence
CGS cloacal gland secretion	OPVL outer perivitelline layer
CM circular manchette	OS Oxidative stress
DIO2 type 2 iodothyronine deiodinase	P4 progesterone
DIO3 type 3 iodothyronine deiodinase	PGF _{2α} Prostaglandin F _{2α}
E2 oestradiol	PVL perivitelline layer
FRF Female Reproductive Fluid	ROS Reactive oxygen species
FSH follicle-stimulating hormone	SSTs Sperm storage structure
GnIH Gonadotropin inhibitory hormone	T testosterone
GnRH Gonadotropins releasing hormone	T3 triiodothyronine
HPG hypothalamic-pituitary-gonadal	T4 thyroxine
IO intromittent organ	ZP zona pellucida

List of figures

Figure 1: Schematic representation of the different last male precedence mechanisms hypotheses in birds

Figure 2: Diagram of a cockerel reproductive tract

Figure 3: Diagram the excurrent ducts of the testis and epididymis ducts

Figure 4: Diagram of a hen reproductive tract

Figure 5: Schematic representation of follicular hierarchy and growth in the hen ovary

Figure 6: Representation of the spermatogenesis process in the Japanese quail

Figure 7: Schematic representation of the spermatogenesis steps

Figure 8: Schematic diagram of spermiogenesis steps in the Japanese quail

Figure 9: Schematic model of neuroendocrine control of seasonal reproduction in birds,

Figure 10: Drawings of the two types of avian sperm morphology

Figure 11: Schematic diagram of physiological polyspermic fertilization in birds

Figure 12: North African houbara bustard female and male performing a display

Figure 13: The Emirates Center for Wildlife Propagation (ECWP) located in Missour, Morrocco.

Figure 14: Schematic summary of the experimental design and methods used for each chapter of the thesis.

Figure 15: Breeding technician collecting a male using a dummy female.

Figure 16: Artificial insemination (AI) procedure done by breeding technicians

Figure 17: Example of results output for SCSA on guavaSoft IMV software.

Figure 18: Schematic representation of kinematics parameters analysed with CASA system

Table I: SCA settings

Table of contents

Introduction.....	12
1. Sexual selection.....	12
1.1 Definition and concepts	12
1.2 Mating systems diversity.....	14
1.2.1 Social monogamy	14
1.2.2 Polygyny	14
1.2.3 Polyandry.....	15
1.2.4 Polygynandry.....	15
1.2.5 Promiscuity.....	15
1.3 Pre-copulatory selection	16
1.3.1 Intra-sexual selection	16
1.3.2 Inter-sexual selection	17
1.4 Post-copulatory selection.....	18
1.4.1 Female cryptic choice.....	18
1.4.2 Male and sperm competition.....	20
a) Post-copulatory male competition.....	20
b) Sperm competition.....	21
c) LMP mechanisms in birds.....	23
2. Aging and reproduction.....	27
2.1 Male reproductive aging	28
2.2 Female reproductive aging.....	30
2.3 Sperm senescence.....	31
3. Avian reproduction.....	32
3.1 Reproductive tract anatomy.....	32
3.1.1 Males	32
3.1.2 Females.....	34
3.2 Folliculogenesis.....	36
3.3 Spermatogenesis	36
3.4 Neuroendocrine control of reproduction.....	41
3.4.1 The Hypothalamus-pituitary-gonadal axis	41
3.4.2 Control of gametogenesis by the neuroendocrine system	44
3.4.3 Control of seasonal breeding	45
3.5 Avian sperm.....	47

3.5.1 Morphology and physiology.....	47
3.5.2 Sperm storage	49
a) In the male tract.....	49
b) In the female oviduct.....	49
3.6 Fertilization.....	51
3.6.1 Egg-sperm interaction and acrosome reaction	52
3.6.2 Polyspermy and syngamy	53
4. The North African houbara bustard	54
4.1 Biology and reproduction.....	54
4.2 Conservation status and captive breeding program	56
4.3 A model species for sexual selection and aging	57
5. Thesis objectives.....	61
Materials and methods	64
1. Houbara conservation breeding.....	65
2. Experimental animals	66
3. Semen collection	66
4. Artificial insemination (AI) procedure	66
5. Egg collection, incubation and chicks rearing	67
6. Blood and tissue collection.....	68
7. Sperm quality analysis.....	68
7.1 Flow cytometry analysis	68
7.1.1 Viability.....	68
7.1.2 Mitochondrial potential	69
7.1.3 Oxidative stress	69
7.1.4 Sperm Chromatin Structure Assay – DNA fragmentation and chromatin decondensation.....	70
7.2 Sperm motility	71
7.3 Sperm morphology	73
8. Reproductive organ collection and histology preparation.....	73
9. Ethics statement.....	74
Results	75
Chapter 1: Sperm morphology, macro and microscopic description of male and female reproductive tract in the North African Houbara Bustard (<i>Chlamydotis undulata undulata</i>).....	75
Chapter 2: Exploring the mechanisms underlying the last male precedence in the North African Houbara Bustard	127
Chapter 3: Testosterone rise precedes the peaks of sexual display and higher sperm quality in adults and old captive North African houbara bustards (<i>Chlamidotys undulata undulata</i>)	154

Chapter 4: Phenotypic trade-offs between sperm quantity and quality across age groups in a polyandrous bird, the North African Houbara Bustard	174
Chapter 5: Pre-but not post-meiotic senescence affects sperm quality and reproductive success in the North African Houbara Bustard	202
Chapter 6: Disentangling the importance of ejaculate attributes, age and mating order in a species with a last male precedence effect	244
Discussion	269
Conclusion and perspectives	282
<i>Résumé en français</i>	285
References.....	303

INTRODUCTION

1. SEXUAL SELECTION

1.1 Definition and concepts

Sexual selection was proposed by (Darwin & Murray 1859) in “On the origin of species by means of natural selection“, and was defined as:

“Sexual selection [...] depends, not on a struggle for existence, but on a struggle between the males for possession of the females; the result is not death to the unsuccessful competitor, but few or no offspring.”

In other words, sexual selection is generated by differential access to opposite-sex mates and fertilization opportunities. Darwin proposed this concept to explain the evolution of certain traits that do not seem to provide any survival or reproductive advantage and are energetically costly to maintain. These traits, called secondary sexual characters, do not directly participate in reproduction, in opposition to primary sexual characters, the reproductive organs. However, secondary sexual characters can give a reproductive advantage by influencing competition to access a mate and mate attraction.

Later in “The descent of man, and selection in relation to sex.” (Darwin 1871), he classified sexual selection in two types:

“The sexual struggle is of two kinds; in the one it is between the individuals of the same sex, generally the males, in order to drive away or kill their rivals, the females remaining passive; whilst in the other, the struggle is likewise between the individuals of the same sex, in order to excite or charm those of the opposite sex, generally the females, which no longer remain passive, but select the more agreeable partners.”

Intra-sexual selection is the competition between individuals of the same sex, usually males, to access the opposite sex, usually females. Inter-sexual selection refers to the mate choice, usually made by the females, depending on the opposite sex, usually males, attractiveness signalled by secondary sexual traits. These traits include all characteristics that can be useful to compete with rivals, such as body mass, strength, and weapons (antlers, horns or tusks), and to attract females, for example, ornaments (colourful plumage or songs) (Andersson & Iwasa 1996).



In most species, males compete for females' access and females choose their mates. Bateman (1948) explained this asymmetry between sexes with a new theory. According to Bateman's principle, females are the limiting resource over which males compete because females almost always invest more energy into producing offspring than males. This concept is based on his experimental results in *Drosophila melanogaster*. Using the same number of males and females, he noticed a difference in reproductive success between the sexes. Almost all females were mating and producing offspring, only four percent did not produce any, whereas many more males, twenty-one percent, were not mating. However, a few males were producing 3 times more offspring than the most fertile female. Therefore, the number of offspring produced by a male depends on the number of females he mated with, whereas one or two matings are enough for a female to fertilize all her eggs. He explained these results by a differential investment of each sex in their gametes. This is called anisogamy: males produce a lot of small gametes, spermatozoa, whereas females produce bigger and more energetically costly gametes, oocytes. Males maximize their reproductive success by producing high sperm number and by fertilizing females, and females' reproductive success is limited by their capacity to produce eggs but not by their ability to fertilize these eggs.

Trivers (1972) added an important concept to explain sexual selection, parental investment. The relative investment of males and females in their offspring controls the way sexual selection operates. When one sex invests more in parental care, such as brooding, nursing, feeding, or protection against predators, individuals of the opposite sex will compete among themselves to access mates. Therefore, parental care influences sexual selection and contributes to the survival and selective value of the offspring. The limiting sex is the one who invests the most in reproduction and parental care and is often the female, but not always depending on the mating system.

Intra and inter-sexual selections occur before copulation and constitute pre-copulatory selection. Working in sperm competition in insects, Parker (1970) introduced a new element of sexual selection occurring after copulation: post-copulatory sexual selection. Similarly to pre-copulatory selection, post-copulatory selection can be divided into two categories: sperm competition and female cryptic choice, equivalent respectively to intra and inter-sexual selection. Inside the female reproductive organs, sperm of different males compete for fertilization (sperm competition) (Parker & Pizzari 2010; Birkhead & Montgomerie 2020) and females can control the likelihood of one male to sire her offspring (female cryptic choice) (Eberhard 2009; Firman *et al.* 2017). One important element to define how post-copulatory sexual selection acts is the mating system. To better understand pre- and post-copulatory selection mechanisms and how they evolved, it is necessary to clarify mating systems. Individuals will invest more in fighting abilities, ornamentation, display or fertility traits for example, depending on the importance of inter-sexual and intra-sexual selection and the risk of sperm

competition of a mating system. Thus, sexual selection will shape the evolution of pre- and post-copulatory mechanisms and traits in differently depending on the mating system.

1.2 Mating systems diversity

Mating systems refer to the pattern of pair bonding, sexual behaviour, and mate number during a reproductive bout of a species. Mating systems influence how pre- and post-copulatory selections will operate and can be classified into five categories explained further.

1.2.1 Social monogamy

Social monogamy is the formation of a pair bond with a single individual of the opposite sex for one breeding season, several ones, or even for a lifetime. The amount of male and female parental care can vary. Monogamy is the most common mating system among birds, and quite rare among other taxa. Approximately 90% of avian species are socially monogamous (Bennett & Owens 2002). However, most socially monogamous species are not genetically monogamous, with females copulating with males outside the pairing, referred as extra-pair copulations, leading to extra-pair paternity (Cockburn 2006; Brouwer & Griffith 2019).

1.2.2 Polygyny

Polygyny is the association of one male with multiple females. There are three main types of polygyny systems:

- Resource defence polygyny where males defend a territory with critical resources needed for successful reproduction (Emlen & Oring 1977).
- Harem defence polygyny where males defend a group of females (Emlen & Oring 1977).
- Lekking systems which are characterised by 4 criteria (Bradbury 1981): (1) males aggregates in specific sites, called “lek”, where they display, (2) there is no male parental care, the investment is limited to copulation, (3) the lek does not provide any critical resource for females and (4) females can choose their mates freely. Classical leks are defined by a high density of male’s aggregation, separated only by a few meters whereas “Exploded leks” corresponds to a less dense aggregation with more distance between males. As no pair bonds exist in lekking system, it can also be defined as a promiscuous system.

1.2.3 Polyandry

In a polyandrous mating system, females' mate or form pair bonds with multiple males. This is the least common mating system in birds. Two main types exist:

- Social polyandry can be divided into two categories: In classic social polyandry, females' mate with several males and lay clutches in separate nests and do not provide any care to eggs and offspring. A sex role reversal is observed with females attracting males and competing for males, and being usually larger, more ornamented and aggressive than males (Johnson & Burley 1998; Kempenaers 2022). In cooperative social polyandry, females form social bonds with multiple males and both female and males give parental care to the offspring at a single nest (Ligon 2000; Kempenaers 2022).
- Resource defence polyandry: females defend a territory and control resources. They mate with several males and lay clutches incubated by each male. Parental care is only provided by males in the system and a sex role reversal is also observed (Emlen & Oring 1977; Oring *et al.* 1994; Johnson & Burley 1998).

1.2.4 Polygynandry

Polygynandry corresponds to larger cooperative breeding units and can be of 2 types (Hauber & Lacey 2005; Kempenaers 2022):

- Classic social polygynandry or plural breeding society is a group of minimum 2 males and 2 females and all individuals are reproductively active.
- Singular breeding is a social unit of males and females with a limited number of breeding individuals, typically a single male and female per group, assisted by non-breeding caregivers' individuals called "alloparents".

1.2.5 Promiscuity

In promiscuous mating systems, there are no pair bond between males and females (Krasnec *et al.* 2012). Only females give parental care. This system often occurs in species with unpredictable environments and usually, it leads to extreme male competition, strong dimorphism (larger males), extravagant ornaments, and displays (Birkhead 2000; Burton 2002).

1.3 Pre-copulatory selection

Pre-copulatory selection is all processes of sexual selection occurring before copulation: competition to access a mating partner, the attraction, and the choice of the mating partner. A sexual dimorphism is observed in many species (Dunn *et al.* 2001; Lindenfors *et al.* 2007; McPherson & Chenoweth 2012), with one sex, usually the male, being bigger and presenting extravagant secondary sexual traits, such as long and colourful feathers and/or song. This ornamentation can be used as signals to compete for access to mates (intra-sexual selection) but also to indirectly signalling quality and attract potential mates (inter-sexual selection) (Andersson & Iwasa 1996).

1.3.1 Intra-sexual selection

The intra-sexual selection is a competition between individuals of the same sex to access and mate with the opposite sex. Usually, males compete between them to mate with females, using strength and weapons and increasing their reproductive success by attracting females, intimidating, and dominating rivals' males (Darwin 1871). A common example of secondary sexual trait used as a weapon and ornamentation is antlers of deers. Male deers use their antlers to visually evaluate rivals and to engage physical competition with these rivals (Gomes *et al.* 2023). Under high levels of intra-sexual selection, antlers size are favoured by selection (Carranza *et al.* 2020). Antlers can also influence female's choice, as shown by Morina *et al.* (2018), females preferred large-antlered males. Male behaviour, through dominance status and aggressiveness, plays an important part in this selection as dominant and more aggressive males are more likely to win the fight against others and access females. This is observed in red jungle fowls (*Gallus gallus domesticus*), males that are more aggressive towards rivals have higher mating success and paternity success (McDonald *et al.* 2017) and in low polyandry groups, dominant males have higher mating success (Collet *et al.* 2012). In species with parental care, competition outcome between males can influence females' choice as it advertises for the ability to protect offspring and access resources. For example, when collared flycatchers (*Ficedula albicollis*) females pair with good competitors, they received better parental care from the male and experienced higher reproductive success than females paired with poor competitors (Qvarnström *et al.* 2000).

Intra-sexual selection can also arise in females competing for male access. For example, in a cooperative breeding group with female dominance, like in the Naked Mole Rat (*Heterocephalus glaber*), only one dominant female produces offspring and represses the breeding of subdominant females through harassment and infanticide (Hauber & Lacey 2005). Female competition can also be observed in cooperative breeding birds, such as the Southern Pied Babbler (*Turdoides bicolor*), where

groups are organized with one dominant female and subordinates' females. Subordinates' females rarely reproduce but, when they do, the reproductive success of the dominant female decreased, less chicks fledged due to nest abandonment and eggs were lost through infanticide by subordinate females. To limit this costs, dominant females are more aggressive toward potential subordinates' females competitors during fertile period (Nelson-Flower *et al.* 2013).

1.3.2 Inter-sexual selection

The inter-sexual selection is defined by the choice of a mate, usually the female choice, based on the opposite sex, usually the male attractiveness signalled by secondary sexual traits, such as ornaments, sexual displays or acoustic signals (Andersson 1994). A well-known example of ornament is the long train of peacock, made of hundreds of feathers with multi-coloured eye like pattern on the feather tip (ocelli) and used for sexual display. Mating success was found to be positively associated with display activity and the number and density of ocelli on the train (Loyau *et al.* 2005). Secondary sexual characters can be honest indicators of a male fertility and parental care. For example, red deer (*Cervus elaphus*) males with large and elaborated antlers have large testes and high sperm motility, and sperm motility is positively correlated with reproductive success in this species (Gomendio *et al.* 2007). In great tits (*Parus major*), males showing larger stripes are more likely to detect predation risk and defend their brood (Norris 1990). However, these ornaments can be energetically costly to maintain and increase predation risk (Kotiaho 2001; Sowersby *et al.* 2022).

Different evolutionary theories have been proposed to explain female's choice:

- Fisher's runaway process: Fisher (1915) proposed a coevolution of females' preference and ornament genes through a self-reinforcing runaway process. When a female preference for a certain ornament increase, both ornament and preference are positively selected. Males possessing this specific ornament have a mating advantage. Females with strong preference will mate with males with extravagant ornaments and therefore a positive selection on the ornament correlated with a positive selection on preference will arise (Andersson 1994). The indirect benefit for the choosy female is that this preference will lead to having more attractive sons that will have a higher mating and reproductive success. This also applies for arbitrary ornaments that do not reflect the quality of the male (Kuijper *et al.* 2012).
- Good genes theory: Female preferences evolved towards male indicators of heritable quality. This heritable quality can be interpreted as higher survival and reproductive value (Kokko *et al.* 2002).



- Compatible genes: The offspring reproductive performance might not reflect the genetic quality of the parents if some epistatic interactions between maternal and paternal genes occur. Therefore, depending on their own genotype, females might select a certain type of males (Kuijper *et al.* 2012).

By being choosy females can obtain direct benefits in terms of survival and fertility (Andersson 1994). The preferences of females can be explained by males advertising for certain qualities like parental care, protection against predators, fertility, or even low parasite load (Barlow 1986; Matthews *et al.* 1997; Møller *et al.* 1999). Female preferences can also evolve in the absence of any direct advantage related to mate choice. Thus, females can make choices to improve the selective values of their offspring such as attractiveness (Fisher 1915), survival (Trivers 1972), avoid inbreeding, and optimize genetic diversity (Zeh & Zeh 1996). Sometimes there is no benefit at all, and female preferences are the result of sensory biases. These sensory biases would have evolved under selection pressure not related to reproduction, such as food foraging or predator avoidance. Males with traits similar to stimuli in which females' perception is favoured by the sensorial bias, would attract more mates and therefore would have greater reproductive success (Kirkpatrick & Ryan 1991).

1.4 Post-copulatory selection

Post-copulatory selection refers to all selection processes occurring during and after mating. It involves various mechanisms driven both by males and females and influencing reproductive success similarly to inter- and intra-sexual pre-copulatory selection. After insemination and before reaching the egg, sperm transit inside the females and can be stored. In most mammalian species the maximum storage time does not exceed a few days, but in other taxa, it ranges from a few months in birds up to years in fishes (up to 4 years) and reptiles (up to 7 years) (Holt & Fazeli 2016). Males will compete by using different strategies to prevent fertilization by another male, sperm of multiple males will compete inside the female tract to fertilize the eggs and females can also have control over which male will sire her offspring.

1.4.1 Female cryptic choice

Females have developed strategies to bias fertilization toward a preferred male. Through this mechanism, called cryptic female's choice (CFC), females can use various mechanisms across taxa such as copulation time, sperm ejection, differential sperm storage, attraction to the egg, or sperm and egg interaction (Eberhard 2009; Firman *et al.* 2017). Sperm ejection is known to occur in birds (Pizzari &



Birkhead 2000; Wagner *et al.* 2004; Dean *et al.* 2011). For example, in feral fowls, females can eject ejaculates of males just after copulation, and they preferentially eject sperm from subdominant males, as the ejection rate was negatively correlated to the social status of the male (Pizzari & Birkhead 2000). In female kittiwakes (*Rissa tridactyla*), sperm from previous inseminations can be ejected to prevent fertilization by senescent sperm (Wagner *et al.* 2004). In insects, the shape and number of storage organs can influence paternity. For example, in *Drosophila melanogaster*, fertilization is biased toward longer sperm when they are stored inside longer seminal receptacle (Miller & Pitnick 2002), and in yellow dung flies (*Scathophaga stercoraria*), females with more storage organs can better control paternity (Ward 2000). The female reproductive tract environment can also modulate fertilization. Female reproductive fluids (FRF) are made of various components such as ovarian, follicular, oviductal or coelomic fluid, egg chemo-attractants, peripheral surfaces to eggs (e.g. egg jelly or cumulus cells, perivitelline layer) and it provides a suitable environment for fertilization (Gasparini *et al.* 2020). In mammals, female reproductive fluids are involved in processes necessary for sperm ability to fertilize an egg, such as sperm capacitation and hyperactivation (Holt & Fazeli 2015). These fluids can also influence sperm quality by increasing sperm motility and velocity in some marine invertebrates (Evans & Sherman 2013), fishes (Rosengrave *et al.* 2016; Zadmajid *et al.* 2019; Myers *et al.* 2020), amphibians (Simmons *et al.* 2009), and mammals (Oliveira *et al.* 1999), and sperm viability in insects (Boer *et al.* 2009; Liberti *et al.* 2016). Evidence of chemical attractants of female reproductive fluids inducing sperm response and attracting sperm toward the egg have been reported in marine invertebrates (Evans & Sherman 2013), fishes (Zadmajid *et al.* 2019; Kholodnyy *et al.* 2020), mammals (Eisenbach & Tur-Kaspa 1994; Fabro *et al.* 2002; Eisenbach & Giojalas 2006; J. Cosson 2015; Fitzpatrick *et al.* 2020). However, the involvement of female reproductive fluids in relation to sperm competition have been rarely investigated. In some fishes biased fertilization success mediated by FRF have been reported. In chinook salmon (*Oncorhynchus tshawytscha*) and masu salmon (*Oncorhynchus masou*), FRF increases sperm velocity of dominant males (Makiguchi *et al.* 2016; Lehnert *et al.* 2017) whereas in ocellated wrasse (*Symphodus ocellatus*), it reduces the number advantage of sneaky males (Alonzo *et al.* 2016). A recent study in the Zebrafish (*Danio rerio*) showed that FRF can select ejaculates with higher sperm number, viability, DNA integrity and resulting in higher fertility (Cattelan *et al.* 2023).

Female reproductive fluids might also be involved in avian species sperm selection. In the Jungle Fowl, females preferentially select sperm from unrelated males in order to avoid inbreeding as more sperm of unrelated males compared to related ones reach the egg (Pizzari *et al.* 2004). In the same species, Løvlie *et al.* (2013) also showed that females can select males based on relatedness. After natural mating, more sperm from males with dissimilar major histocompatibility (MHC) genes, were found on the egg membrane compared to sperm from similar MHC. However, this advantage



was lost when females were artificially inseminated, suggesting selection against relatedness is not only controlled by female cryptic choice but also signalled by phenotypic traits during pre-copulatory selection (Løvlie *et al.* 2013). Females can also influence the pronuclei fusion, the karyogamy. In the Comb Jelly (*Beroë ovata*), an invertebrate species, physiologic polyspermy occurs, meaning that several sperm enter the egg, and pronuclei fusion is not random as the female pronucleus does not necessarily fuse with the first sperm pronucleus encountered. Indeed in most of the cases, (Carré & Sardet 1984) observed, with time-lapse microscopic video recordings, the female pronucleus migrated toward a sperm pronucleus and then a reversion of its trajectory and migration toward another pronucleus occurred. They also reported up to three migrations before pronuclei fusion occurred.

1.4.2 Male and sperm competition

a) Post-copulatory male competition

During and after copulation, males can adopt different strategies to outcompete rivals and maximise chances of fertilization (Parker 1970; Eberhard 2009). In birds, males can prevent females to copulate with other males and therefore limit the risk of sperm competition by guarding their mate and copulate frequently (Møller & Birkhead 1991; Harts *et al.* 2016). Mate guarding behaviours and frequent copulations are usually more frequent during the female fertile period (Barber & Robertson 2007; Hoi *et al.* 2011). For example, in house sparrows (*Passer domesticus*), males start following their paired female 7 days before the first egg is laid and this behaviour lasts until the clutch is completed (Hoi *et al.* 2011). In tree swallows (*Tachycineta bicolor*), as much as 18 attempts of copulations per hour can be observed during the fertile period (Crowe *et al.* 2009). These strategies are more frequently observed in polyandrous species with high risk of sperm competition compared to polygynous ones (Møller & Birkhead 1991). Mate guarding is also frequent among insect taxa and they also increase copulation time to prevent mating with other males (Parker 1970). For example, in the House Fly (*Mus domestica*) sperm is transferred within 15 minutes but copulation lasts about an hour (Murvosh *et al.* 1964). Another common strategy used to limit sperm competition and secure paternity in insects is the removal of stored sperm inside the female storage organ by the male genitalia (Simmons *et al.* 1996; Córdoba-Aguilar *et al.* 2003; Kamimura 2005; Nakahara & Tsubaki 2007; Cordero-Rivera 2017). A few bird species present a male intromittent organ (IO) that is inserted into the female cloaca during copulation and used to deposit sperm inside the female reproductive tract (Briskie & Montgomerie 1997). Thus, IO allows sperm to be deposit deeper inside the female oviduct than it would be with cloacal transfer, reducing the probability of sperm ejection by the female (Pizzari & Birkhead 2000). The possibility of previously stored sperm removal by IO, similarly to insects, have been suggested before, however direct evidence of such process have never been reported up to date

(Briskie & Montgomerie 1997; Coker 1998; McKone 2023). Moreover, male's ejaculate can coagulate and form a mating or copulatory plug in the female genital to prevent copulation and insemination from other males; this strategy has been documented in insects, mammals and reptiles (Schneider *et al.* 2016).

b) Sperm competition

After insemination and before reaching the egg, sperm transit inside the females and can be stored. In most mammalian species the maximum storage time does not exceed a few days, but in other taxa, it ranges from a few months in birds up to years in fishes (up to 4 years) and reptiles (up to 7 years) (Holt & Fazeli 2016). When females mate with multiple males, their sperm will compete for fertilization of the ovum. Sperm traits play an important part in the outcome of this competition as they can predict fertilization success (Pizzari & Parker 2009).

According to the “raffle principle”, the probability of fertilization success of a male equals his number of sperm inseminated divided by the total number of sperm inside the female tract when all other sperm traits are equivalent (“fair raffle”). Sperm number is indeed a strong predictor of fertilization success and a positive correlation between number and fertility has been observed in many species, in insects (Hale *et al.* 2008), fishes (Stoltz & Neff 2006; Boschetto *et al.* 2011), birds (Martin *et al.* 1974; Saint Jalme *et al.* 1994; Liu *et al.* 2008; Sorci *et al.* 2023) and mammals (Yaniz *et al.* 2002; Belstra *et al.* 2020).

However, when sperm traits, other than number, differ between competitive males, it is not a “fair raffle” anymore but a “loaded raffle” with one male having an advantage over the other. Thus, other sperm traits can influence fertilization and reproductive success. Sperm motility and velocity are associated with higher fertilization success in birds (Froman *et al.* 1999; Birkhead *et al.* 1999; King *et al.* 2000; Denk *et al.* 2005), fishes (Lahnsteiner *et al.* 1998; Gage *et al.* 2004; Liljedal *et al.* 2008; Boschetto *et al.* 2011), mammals (Malo *et al.* 2005; Gomendio *et al.* 2007; Broekhuijse *et al.* 2012; Kummer *et al.* 2013; Mocé *et al.* 2022) and invertebrates (Levitan 2000; Kupriyanova & Havenhand 2002). Sperm viability, the proportion of live sperm cells in the ejaculates, can also predict fertilization outcome (Hunter & Birkhead 2002; García-González & Simmons 2005; Smith 2012; Chen *et al.* 2016). Sperm morphology, the relative proportion of sperm presenting a normal morphology and defects, and morphometry, the variation of sperm phenotypes (sperm compartments or full sperm length) are also associated with fertility outcome. Sperm length can be positively or negatively correlated with fertilization success, in birds longer sperm is usually associated with higher fertility (Briskie *et al.* 1997; Bennison *et al.* 2015) whereas in some insects males with shorter sperm have a better fertilization



success (García-González & Simmons 2007). Positive correlations between proportion of normal sperm morphology and fertilization success have been reported in humans (Lukaszewicz *et al.* 2008; Enginsu *et al.* 1991; El-Ghobashy & West 2003; Franken & Henkel 2012; Shabtaie *et al.* 2016), other mammals (Malo *et al.* 2005; Gomendio *et al.* 2007; Al-Makhzoomi *et al.* 2008; Love 2011), and birds (Łukaszewicz 1988). Sperm DNA integrity is mandatory for successful fertilization and embryonic development and therefore DNA damage can impair reproductive success. In humans (Casanovas *et al.* 2019; Ribas-Maynou & Benet 2019; Ribas-Maynou *et al.* 2021), other mammal species (Simões *et al.* 2013; Gawecka *et al.* 2013; Kumaresan *et al.* 2020; Mateo-Otero *et al.* 2022), and fishes (Pérez-Cerezales *et al.* 2010) a negative impact of DNA damage have been reported on *in vivo* and *in vitro* fertility outcome. A recent study also suggested that DNA damage caused by oxidative stress impairs early embryo development in pigs (Mateo-Otero *et al.* 2024). Moreover, oxidative stress and reactive oxygen species (ROS) production by sperm can also impact fertility, by inducing membrane oxidation, DNA damage, reducing motility and impairing fertilization (Treulen *et al.* 2015; Aitken 2020; Boguenet *et al.* 2021). Therefore, sperm traits play an important role in the outcome of sperm competition. When males are exposed to a high risk of sperm competition, they are expected to produce high-quality sperm to secure paternity. Thus, in polyandrous species, males tend to have larger testis and produce high-quality sperm therefore being more competitive (Lüpold *et al.* 2011).

Sperm quality is not the only factor favouring siring success, the order in the mating sequence is also important. Usually, the male in last position in the mating or insemination sequence has an advantage on fertilization and sire most offspring. This phenomenon is called Last male precedence (here after LMP) and is widespread among insects and birds (Parker 1970; Birkhead & Montgomerie 2020). Indeed this last male advantage was observed in several avian species including domestic fowls (*Gallus gallus domesticus*) (Warren & Gish 1943; Compton *et al.* 1978), turkeys (*Meleagris gallopavo*) (Payne & Kahrs 1961; Christensen 1981), emus (*Dromaius novaehollandiae*) (Taylor *et al.* 2000), ducks (Cheng *et al.* 1983; Stai & Searcy 2010), ringed turtle doves (*Streptopelia capicola*) (Sims *et al.* 1987), houbara bustards (Sorci *et al.* 2023) and passerines birds (Zebra Finch: (Birkhead *et al.* 1988), Yellow-headed Blackbird (*Xanthocephalus xanthocephalus*): (Briskie 1996), and study reported from 60 to 80% percent of offspring attributed to the last males. This range is likely due to the different experimental conditions applied because different time intervals have been used and females were not always in the same reproductive stage (delay before first egg laying). Although different hypotheses have been proposed to explain this phenomenon, there is no consensus on the mechanism, which is likely to be species dependent.

c) LMP mechanisms in birds

Mechanisms underlying this fertilization advantage have been explored in insect species and sperm displacement by sperm of the last male inside the female storage structure occurs in the drosophila (Price *et al.* 1999; Manier *et al.* 2010). In birds, five different mechanisms explaining LMP have been proposed by (Lessells & Birkhead 1990; Briskie 1996; Birkhead & Biggins 1998). They built mathematical models based on their hypothesis and applied them to several experimental data in different avian species. These models were based on paternity outcomes and the models' predictions were confronted with observed paternity results. Other studies used a different approach to assess sperm competition and used techniques to directly visualize sperm inside sperm storage tubules (SSTs), with radioactive labelling (van Krey *et al.* 1981), with a fluorescent labelling (King *et al.* 2002; Matsuzaki *et al.* 2023) or by using sperm from males with different phenotypes (Hemmings & Birkhead 2017). The following sections will present the different hypotheses and the experimental data associated.

- Passive sperm loss over time

The first mechanism proposed to explain LMP was a passive sperm loss over time and was proposed by (Lessells & Birkhead 1990) (Fig.1 A). After one insemination or mating, a fertility decrease is observed over time and is due to a decrease in sperm number stored inside SSTs (Wishart 1987; Briskie & Birkhead 1993). Therefore, the last male in a mating sequence would have an advantage in number over previous ones. This hypothesis is believed to be the most important mechanism explaining the last male precedence. Experimental data were in agreement with this model predictions in the Turkey and the Domestic Fowl (Birkhead & Biggins 1998).

- Sperm stratification

According to this hypothesis, sperm of different males will stratify inside SSTs and will not mix (Birkhead & Biggins 1998) (Fig.1B). This will result in sperm from the first mating/insemination being stored at the bottom of SSTs whereas sperm from more recent matings will be stored closer to SSTs opening, therefore, will be closer to the fertilization site. The sperm storage pattern inside SSTs was studied in passerines and stratification of sperm was observed in 18 species (Briskie & Montgomerie 1993). Other studies suggested a stratification inside the SSTs in the Turkey (Christensen 1981) and in the Chicken (Compton *et al.* 1978); however none of these studies used techniques to differentiate sperm from different males.

- Sperm displacement

Sperm from a male in the last position in the mating sequence will actively remove stored sperm from previous matings (Birkhead & Biggins 1998) (Fig.1C). Using a combination of radiolabelled (3H-thymidine) and unlabelled sperm in fowls, van Krey *et al.* (1981) found no evidence of sperm displacement. Birkhead and Biggins (1998) tested their mathematical model for sperm displacement in turkeys and hens and failed to find displacement evidence. Finally, more recently, Matsuzaki *et al.* (2023) used a double fluorescent sperm labelling to investigate LMP in fowls. After two consecutive inseminations with a three-day interval, more than 70% of sperm from the first insemination was replaced by sperm from the second one. They concluded that sperm displacement was likely to be the most important mechanism of LMP in chickens. However, they also reported evidence of sperm loss with a decreased stored sperm number over the first five days after insemination.

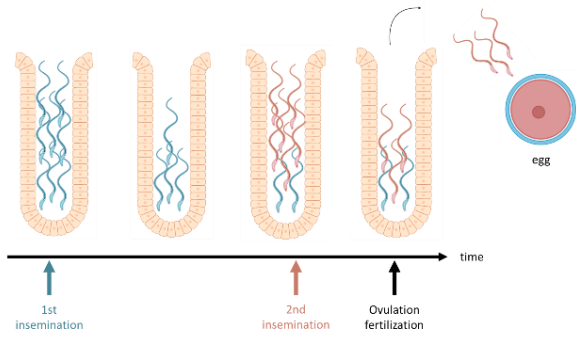
- Sperm segregation

Spermatozoa of different males will be stored in separate SSTs (Fig.1D). King *et al.* (2002) found evidence of sperm segregation in turkeys and chickens. They used sperm stained with Hoechst 3333 and unstained to differentiate samples from different inseminations and they observed only 4% and 12%, respectively in chickens and turkeys, of SSTs filled with a mixed population of sperm. In zebra finches (*Taeniopygia guttata*), segregation was also observed, with 99.8% of tubules observed containing only sperm from one or another successive matings based on sperm phenotype (long and short sperm) (Hemmings & Birkhead 2017).

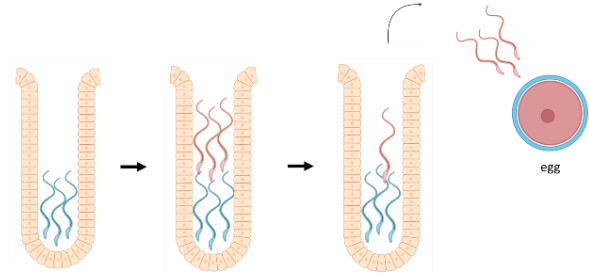
- Spatio-temporal filling

Finally, another potential mechanism proposed is a spatio-temporal filling (Fig.1E, (Briskie 1996)): SSTs closer to the vagina will be filled first and those located closer to the uterus, and thus closer to the fertilization site, will be filled later with sperm from a more recent insemination, thus giving males in the last position of the sequence a fertilization advantage. Therefore, this hypothesis also implies sperm segregation where sperm from different inseminations will not mix within the same tubule but will be stored in different ones. This mechanism was explained by a different spatio-temporal maturation of SSTs. Indeed, SSTs closer to the vagina would mature and store sperm earlier than those closer to the uterus. Evidence of this hypothesis was found in the Yellow-headed Blackbird (*Xanthocephalus xanthocephalu*) (Briskie 1996), where females showed a sequential maturation of their SSTs leading to a differential sperm storage.

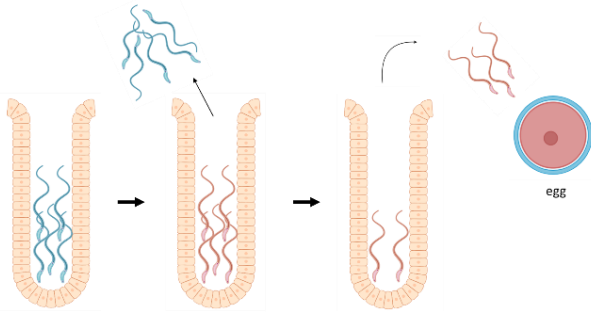
A Passive sperm loss



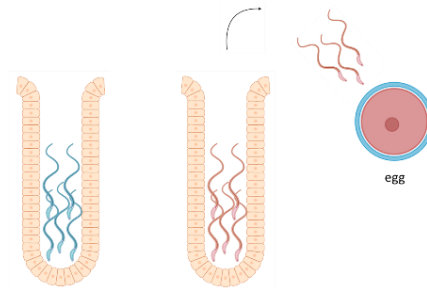
B Sperm stratification



C Sperm displacement



D Sperm segregation



E Spatio-temporal filling

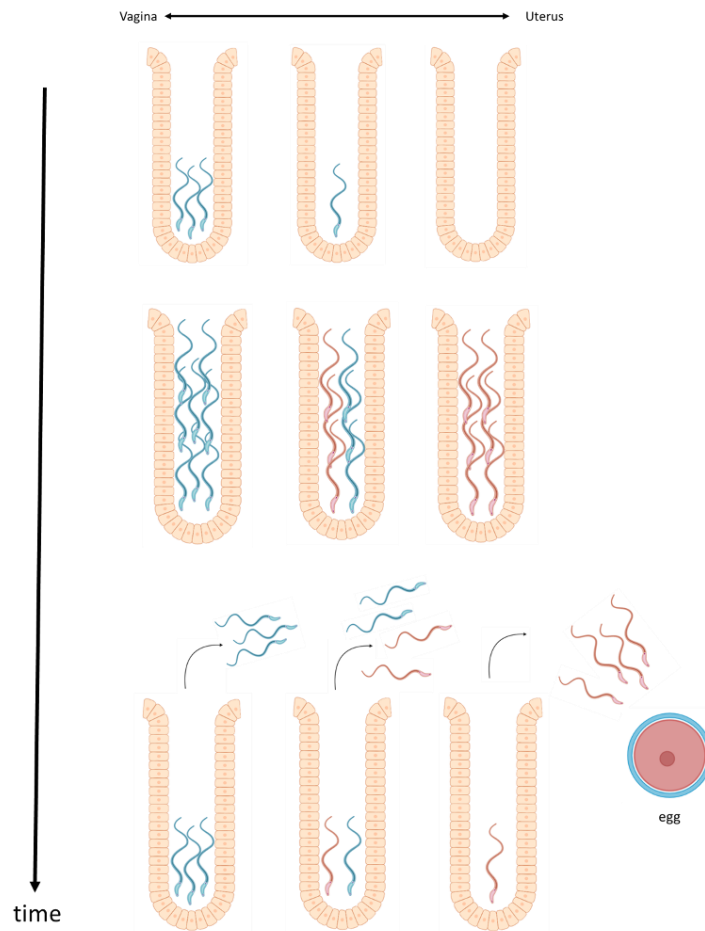


Figure 1: Schematic representation of the different last male precedence mechanisms in birds: A) The passive sperm loss model: The number of sperm stored from a first insemination (red slope) declines with time so sperm from a second insemination (blue) have an advantage in number; B) Sperm stratification: Sperm of different inseminations are stratified in layers inside the same SSTs, sperm from the first insemination (blue) are located at the bottom of the tubule whereas sperm from the last one are stored closer to the opening of the tubule and to the fertilization site; C) Active sperm displacement: sperm of the last male (red) remove sperm already stored in SSTs (blue); D) Sperm segregation: sperm of different inseminations are stored in different SSTs and not mixed within the same tubules. E) Spatio-temporal filling: SSTs closer to the vagina are filled first by sperm of first inseminations (blue) whereas SSTs closer to the uterus and fertilization site are stored by sperm from the last insemination (red). (Created with BioRender.com)

Although passive sperm loss is believed to be the main strategy responsible for the last male precedence, there is no clear pattern emerging from these different studies. Most of these experiments used mathematical models to compare the model's predictions with observed paternity outcomes. Only a few studies used techniques to visualize sperm inside the storage structure and such techniques are necessary to further explore storage mechanisms underlying LMP in birds.

In addition to sperm quality and mating order, age can also bias reproductive outcomes. Indeed, age related impairment on reproduction has been reported in many species (Nussey *et al.* 2013; Lemaître & Gaillard 2017) and it can impact both pre-copulatory and post-copulatory selection. Indeed, older males can show impaired expression of secondary sexual traits, making them less attractive to females and/or less competitive than other males, leading to reduced chances of mating. For example, in red jungle fowls, older males have smaller comb (Cornwallis *et al.* 2014) and under high competition conditions, in male biased groups, they are less likely to be dominant and to mate with females (Dean *et al.* 2010). Post copulatory selection is also impacted in this species, as shown by a decrease of sperm quality with age (Cornwallis *et al.* 2014) and a decrease of fertilization success (Dean *et al.* 2010). Ultimately, by affecting pre- and post-copulatory selection, age can have a negative impact on reproductive success and fitness.

2. AGING AND REPRODUCTION

Senescence or aging can be described as a decline of physiological, hormonal, and cellular functions with age resulting in survival and reproductive success decrease (Monaghan *et al.* 2008). Aging is often described as an evolutionary paradox. Aging is a deleterious trait as it impairs health, fertility and survival of an organism; however, senescence is widespread among animals. As natural selection operates to select individuals for optimal survival and reproduction, it has always been questioned why and how such detrimental trait has evolved (Fabian & Flatt 2011).

Three main theories were proposed to explain the evolution of aging (Kirkwood & Austad 2000). All these theories are based on the theoretical fundamental concept of the decline of the strength of natural selection with age. The strength of natural selection corresponds to a measure of how strongly selection acts on survival and reproduction (Hamilton 1966; Rose *et al.* 2007). According to the first theory, the mutation accumulation theory (Medawar 1952), deleterious mutations that are expressed late in life will accumulate across generations. Indeed, when the negative effect of these mutations appears in late life, the carriers would have already reproduced and passed on their genes to the next generation, and natural selection would be weak and unable to eliminate such mutations. The impact of this mutation's accumulation process would become visible at the organismal level when individuals live long enough to show aging effects (Fabian & Flatt 2011). The second one, the antagonist pleiotropy theory, was proposed by Williams (1957). According to this theory, aging is the consequence of antagonistic pleiotropic genes. These genes have beneficial effects in early life, but negative ones in late life. These genes would be favoured in early life when the force of selection is strong even if the same genes have deleterious effects late in life. These two theories are not mutually exclusive, and both mechanisms can operate at the same time (Le Bourg 2014). The third theory is the disposable soma proposed by Kirkwood (1977). According to this theory, aging arises because of an energetic trade-off between reproduction and somatic maintenance. Somatic maintenance includes all mechanisms not related to reproductive function such as body maintenance, immunity, and cellular repair system. Because of limited resources, investment of energy will be higher in reproduction and lower in soma maintenance leading to senescence.

These three theories predict that if extrinsic risk of mortality is high, organisms will invest more in reproduction instead of body maintenance, thus individuals living longer than the average lifespan will experience aging. If extrinsic risk of mortality is low, individuals will live longer and therefore will invest more in body maintenance than in reproduction and have a better healthy life span (Johnson *et al.* 2019).



Reproductive senescence is the specific deterioration of reproductive functions leading to a decrease in fitness. Indeed, aging is generally associated with a decline in reproductive organs and endocrine system functions, and in gamete quantity and quality (Noguera *et al.* 2012; Gunes *et al.* 2016; Fricke & Koppik 2019). All of the declines are in the end impairing fertilization success, embryo development, and offspring quality (Hale *et al.* 2008; Kim *et al.* 2011; Lippens *et al.* 2017; Lemaître & Gaillard 2017; Ruhmann *et al.* 2018). Both male's and female's fertility can be impacted by reproductive aging with consequences on pre- and post-copulatory sexual selection.

2.1 Male reproductive aging

Hormonal imbalance of androgens and gonadotropins can be observed in aging males, this has been evident in humans, where gonadotropins levels, like follicle-stimulating hormone (FSH) and luteinizing hormone (LH), tend to increase whereas circulating testosterone decreases, impacting testicular function and sperm production. These hormonal changes are associated with a decrease of testicular volume, number of germ cells, Leydig and Sertoli cells, and impaired spermatogenesis leading to reduced sperm production (Neaves *et al.* 1984; Dakouane *et al.* 2005; Gunes *et al.* 2016). Testosterone level decrease can also impact sexual behaviour; senescent Japanese quail (*Coturnix japonica*) males have decreased circulating androgen levels and show a progressive loss in the frequency and intensity of courtship and mating behaviours (Ottinger 2010). A decrease in mating success is also observed in fowls with old males being less aggressive which makes them less competitive with other males resulting in fewer copulations (McDonald *et al.* 2017).

A sperm quality impairment related to age has been observed among many animals including mammals, birds and fishes, with old males often having a lower ejaculate volume and sperm number ejaculated (Wolf *et al.* 2000; Sartorius & Nieschlag 2010; Preston *et al.* 2011; Gunes *et al.* 2016; Johnson *et al.* 2018), a decline in sperm motility (Preston *et al.* 2011; Johnson *et al.* 2018) and viability (Chantepie *et al.* 2015; Gunes *et al.* 2016), an increase in ROS production and a decrease of antioxidants levels (Noguera *et al.* 2012). Reactive oxygen species (ROS) are free radicals containing unpaired electrons that are very reactive toward sugars, lipids, proteins, or nucleic acids (Aprioku 2013). ROS are produced in physiological conditions by cells but can also come from exogenous sources in the environment, such as chemicals or drugs (Møller *et al.* 2014; Aitken *et al.* 2014). Mitochondria produce ROS during oxidative phosphorylation, and they are necessary mediators to maintain cellular homeostasis, being involved in sperm capacitation, acrosome reaction, and fertilization in some species (Koksal *et al.* 2003; Lamirande & Lamothe 2009; Aitken & Curry 2011). Oxidative stress without antioxidant balance, may ultimately lead to mitochondrial dysfunction, apoptosis and DNA damage (Reinhardt 2007; Aitken *et al.* 2014). Levels of antioxidants in seminal plasma can also be impacted by



aging. In fowls, reduced levels of seminal antioxidants were associated with reduced sperm motility and oxidative sperm DNA damage (Noguera *et al.* 2012).

The testis tissue also ages, and a decline of the spermatogenetic activity is observed. Apoptosis is a normal process during spermatogenesis, however with age, the balance between germ cells apoptosis and spermatogonia proliferation is affected (Pastor *et al.* 2011). In old men, an apoptosis increase, and proliferation decrease in germ cells were observed, as well as a deterioration of cells that are supporting spermatogenesis, the Sertoli cells (Kimura *et al.* 2003). Moreover, male germ cells undergo continuous divisions across the reproductive lifespan of an individual, leading to the accumulation of mutations with age (Yoon *et al.* 2009). Besides mutation, sperm DNA integrity can also be impaired by oxidation and fragmentation. Sperm cells are prone to DNA damage as they lack a DNA repair mechanism (González-Marín *et al.* 2012). Higher DNA damage levels have been reported in old men (Wyrobek *et al.* 2006; Kong *et al.* 2012), in the Red Jungle Fowl (Noguera *et al.* 2012) and in old blue-footed booby males (Velando *et al.* 2011).

DNA integrity of sperm cells is a key parameter for successful fertilization and the development of a healthy embryo and offspring through lifetime. Therefore, this decline of the genetic quality of sperm, from old males can have consequences and lead to failed fertilization, poor quality of embryo and embryonic death, or even transgenerational effects with consequences on the offspring's health and their germlines. Advanced paternal age in men is associated with decreased pregnancy rate, increased spontaneous abortions and miscarriages, congenital abnormalities, and neurodevelopmental disorders (Sharma *et al.* 2015). Mating and fertilization success decrease with age in the Red Jungle Fowl (Dean *et al.* 2010; Carleial *et al.* 2020). Evidence of a decrease in fertilization success and an increase in embryo/offspring mortality was also found in insects (Hale *et al.* 2008; Ruhmann *et al.* 2018), birds (Dean *et al.* 2010; Kim *et al.* 2011; Fay *et al.* 2016; Carleial *et al.* 2020) and nematodes (Lippens *et al.* 2017). Epigenetic marks include DNA methylation, histone modifications and non-coding RNA profiles and modulate gene expression without modification of the DNA sequence. The sperm epigenome can be impacted by age in mammals, and these changes can target gene networks involved in embryo developmental, neurodevelopmental, growth and metabolic pathways (Ashapkin *et al.* 2023) and overall, these epigenetic age related signatures can alter the offspring epigenome and phenotype (Denomme *et al.* 2020; Ashapkin *et al.* 2023; Dahlen *et al.* 2023).

2.2 Female reproductive aging

Birds and mammals were believed to have a finite supply of primary oocytes that decreases with age unlike most insects, amphibians, and reptiles. For example, in women between 16 and 20 days of gestation, foetal ovaries reach a peak of 7 million of primordial follicles, at birth only 1 to 2 million remains, and at puberty the number falls to 400 000-500 000 primordial follicles. During her reproductive life, a woman will ovulate around 400 oocytes and at menopause only 1000 primordial follicles would remain (Secomandi *et al.* 2022).

However, over the past decade this dogma of a finite ovarian reserve has been questioned with new studies showing the existence of oogonial stem cells in mammals (Gosden 2004; Johnson *et al.* 2004; White *et al.* 2012; Sequeira *et al.* 2021) and recently in chicken ovary (Meng *et al.* 2023). Nevertheless, there is an age-related depletion of the ovarian reserve in aging mammalian females. A depletion of primary oocyte is not always observed in birds, it is the case in Japanese quails but not in budgerigars (*Melopsittacus undulatus*), so the depletion of primary oocyte reserve is not the only cause of fertility decline with age (Holmes & Ottinger 2003).

Not only the quantity but also the quality of oocyte decreases with age (Secomandi *et al.* 2022). Cellular mechanisms of oocyte senescence are multiple. With age there is an accumulation of advanced glycation end products (AGEs); these molecules, produced by non-enzymatic glycation reactions, are pro-inflammatory (Tatone *et al.* 2011). Their accumulation in the ovary causes a reduced oocyte developmental capacity and causes protein damage, oxidative stress (OS) reaction and inflammation (Pertynska-Marczewska & Diamanti-Kandarakis 2017). OS is also needed in the last step of AGEs production, making these molecules both a source and a consequence of OS. ROS levels also increase in the mammalian ovary with age, oocyte produces less antioxidant. OS increases in the granulosa cells and is associated with abnormal follicular function due to disruption of the hormonal signalling pathway. OS increases can also lead to mitochondrial dysfunction and apoptosis. DNA damage accumulates with age as DNA repair mechanisms are impaired (Secomandi *et al.* 2022).

Telomere shortening is another aspect of age-related fertility decline in female mammals. Telomere attrition can be caused by oxidation due to high ROS levels and by a reduction of telomerase activity (Kosebent *et al.* 2018). Telomere length is important for chromosome alignment and meiotic spindle during meiosis to prevent aneuploidy of embryos (Kalmbach *et al.* 2013).

As for sperm, oocyte quality and DNA integrity are mandatory for successful fertilization and to ensure embryo development and the offspring's health. Thus, decreasing oocyte quality can lead

to fertilization and embryonic development failure, abortion, and birth defects (Kalmbach *et al.* 2013; Harris *et al.* 2017; Cimadomo *et al.* 2018; Frick 2021).

Hormonal changes are also observed in aging females. The ovarian function decline, in aging women, is associated with lower oestradiol serum levels and higher FSH concentrations compared to younger women (Chahal & Drake 2007). In Japanese quails, a decrease of circulating progesterone and FSH is observed impacting folliculogenesis, follicular growth is reduced, and the number of primary oocytes decreases, there is oviduct atrophy and LH sensitivity decrease, however, there is little modification of oestrogen levels (Holmes & Ottinger 2003).

2.3 Sperm senescence

Independently from male age, sperm can also be affected by cellular senescence. Indeed, during transit and storage inside the male ducts but also inside the female oviduct, sperm cells age. Due to their specific characteristics, spermatozoa are prone to oxidative damage (Reinhardt 2007). Spermatozoa contain numerous mitochondria and therefore produce high amount of ROS; they have a limited antioxidant defence due to their small amount of cytosol and their membranes are very rich in polyunsaturated fatty acids and thus highly prone to oxidation. Additionally, spermatozoa also lack a DNA repair system (González-Marín *et al.* 2012; Aitken 2020). Oxidative stress can compromise sperm motility, viability and DNA integrity (Tarín *et al.* 2000). Sperm quality decrease can impair fertilization success and offspring's health; in the Guppy (*Poecilia reticulata*), sperm senescence resulted in a decrease in sperm velocity and fertilization success (Gasparini *et al.* 2014; Gasparini *et al.* 2017). A reduced sperm viability and increased DNA fragmentation were associated with prolonged sperm storage in the Zebrafish (Cattelan & Gasparini 2021). In kittiwakes (*Rissa tridactyla*), senescent sperm impaired fertilization, embryo development, hatching, and chick's condition (White *et al.* 2008).

However not all species are affected by reproductive senescence in the same way and a wide range of aging patterns can be observed in animals: from rapid death just after a single breeding season, called semelparity observed in some salmonids, marsupials, and insects to almost inexistent reproductive aging like in naked mole rats (Comizzoli & Ottinger 2021). In some species, reproductive success even increases with age, this pattern can be observed in several birds with extra pair paternity (Lifjeld *et al.* 2022) and older males usually have higher extra pair fertilization success (Hsu *et al.* 2015; Kaiser *et al.* 2017; Girndt *et al.* 2018), bigger testis and produce more sperm of better quality (Birkhead *et al.* 1997; Graves 2004; Laskemoen *et al.* 2008).

Therefore, to study reproductive aging and its potential impact on sexual selection, knowledge on the characteristics and specificities of a model species is important, and studying reproductive



biology is mandatory. For example, in the context of sperm competition, to study the sperm storage dynamics and by which mechanism the last male in the mating sequence has an advantage in fertilization, spermatozoa inside the storage structures must be observed. For that, the anatomic location of the storage structures must be known. Moreover, collection and observation methods must be mastered to allow consistent analysis. However, this cannot be done without any knowledge of reproductive biology and especially of anatomy.

As the main aim of this thesis work is to explore reproductive aging and sexual selection in a wild polyandrous bird, the North African Houbara Bustard (*Chlamydotis undalata undulata*), the following section will focus on avian reproductive biology.

3. AVIAN REPRODUCTION

To better understand the mechanisms of sexual selection and reproductive aging, knowledge of reproductive biology is essential. While there are over 10,000 known avian species, the reproductive biology of birds has only been well-documented for a few model species of domestic birds or species with commercial interest, such as poultry (chickens, turkeys, and quails) and ratites (ostriches (*Struthio camelus*), emus, and rheas (*Rhea americana*)). Knowledge is still lacking in some areas. This is particularly true for wild birds, where the literature is scarce and only a few species have been studied. The following sections provide a brief overview of avian reproductive biology mainly based on poultry models.

3.1 Reproductive tract anatomy

3.1.1 Males

Males have two intra-coelomic bean or ova shaped testis in ventral position to the vertebral column and attached to the dorsal cavity wall by a mesorchium, two epididymis and two deferent ducts (Fig 2). The testis consists of seminiferous tubules, lined by a spermatogenic epithelium, and interstitial space containing blood vessels and Leydig cells involved in androgen production. This seminiferous epithelium and interstitial cells are surrounded by a testicular capsule usually thin in most avian species but sometimes thicker like in emus (Du Plessis 2012). The epididymis is attached at the dorsomedial surface of the testis and consists of a series of ducts: the rete testis, connected to seminiferous tubules, efferent ducts, connecting ducts, and finally one epididymal duct (Fig. 3). The caudal end of the epididymis is continued by a tortuous deferent duct that opens in the cloaca. In sexually active birds, testes are usually white to yellow coloured but some species, such as ratites (Fowler 1991; Du Plessis 2012), cockatoos, mynahs, and toucans (Taylor 1989) can have pigmented

testes. In most birds, testes are asymmetrical, with the left testis larger than the right, and in case of seasonal species, a regression of both testis is generally observed during the non-breeding season as well as a change in colour for species with pigmented testes. During the breeding period, testis size can increase in size up to 500 folds (King & McLelland 1984).

Only 3% of known bird species have an intromittent phallus (Herrera *et al.* 2013), such as ratites, tinamous and birds of the Anseriformes order. Other birds, including domestic fowls, turkeys and some passerines, do not present an intromittent organ and in this case, during mating sperm is transferred via the contact of male and female cloaca (King & McLelland 1984).

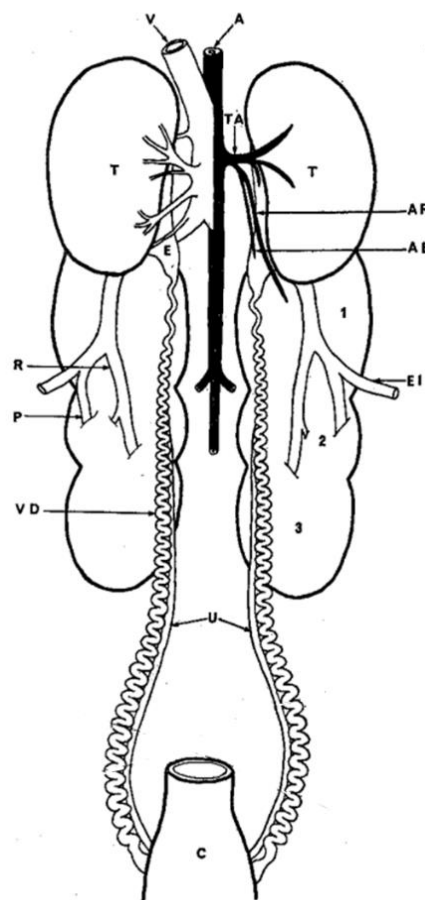


Figure 2: Diagram of a cockerel reproductive tract (From Hodges 1974) : A: Abdominal aorta; AE: Epididymal artery; AR: anterior renal artery; C: cloaca; E: epididymis; EI: external iliac vein; P: caudal renal portal vein; R: renal vein; T: testis; TA: testicular artery; U: ureters; V: posterior vena cava with testicular veins from the right testis draining into it; VD: Vas deferens; One side of the vena cava/testicular vein complex is not represented in order to demonstrate the underlying arterial supply to the left testis: 1, 2 and 3: anterior, middle and posterior divisions of the left kidney.

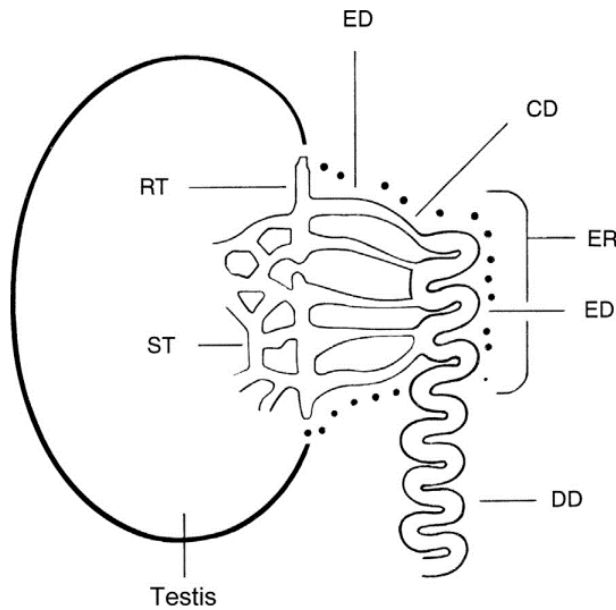


Figure 3: Diagram the excurrent ducts of the testis and epididymis ducts (From Vizcarra *et al.* 2022): ST: seminiferous tubules, RT: rete testis, ED: efferent duct, CD: connecting duct, ER: epididymal region, ED: epididymal duct, DD: deferent duct.

3.1.2 Females

Except some raptors species or the Brown Kiwi, that have both right and left ovaries developed and functional (King & McLelland 1984), in most females' birds only the left ovary is functional and the right ovary regresses during embryonic development. The avian oviduct is composed of five segments: the infundibulum, the magnum, the isthmus, the uterus, and the vagina (Fig. 4). The infundibulum consists of two regions: the funnel region, where sperm egg encounter occurs, and the tubular region also called the chalaziferous zone. The later region secretes the chalaziferous layer of the albumen that surrounds the ovum. In chickens (Hrabia 2022) the egg passes through the infundibulum within 15-30 minutes. When it arrives in the magnum, albumen is secreted by tubular glands of the mucosa and added to the egg. After three hours, the egg arrives inside the isthmus where a small amount of albumen proteins is added, inner and outer shell membranes are formed, and the calcification of the shell is initiated. The egg transits inside the isthmus for approximately 75 to 90 minutes after which it enters the uterus (or shell gland). For about 20 hours, the calcareous shell is deposited, and the hard-shelled egg passes through the vagina and is then expelled through the cloaca. The total time between ovulation and egg position ranges from 24 to 28 hours in chickens (Pollock & Orosz 2002; Johnson 2015).

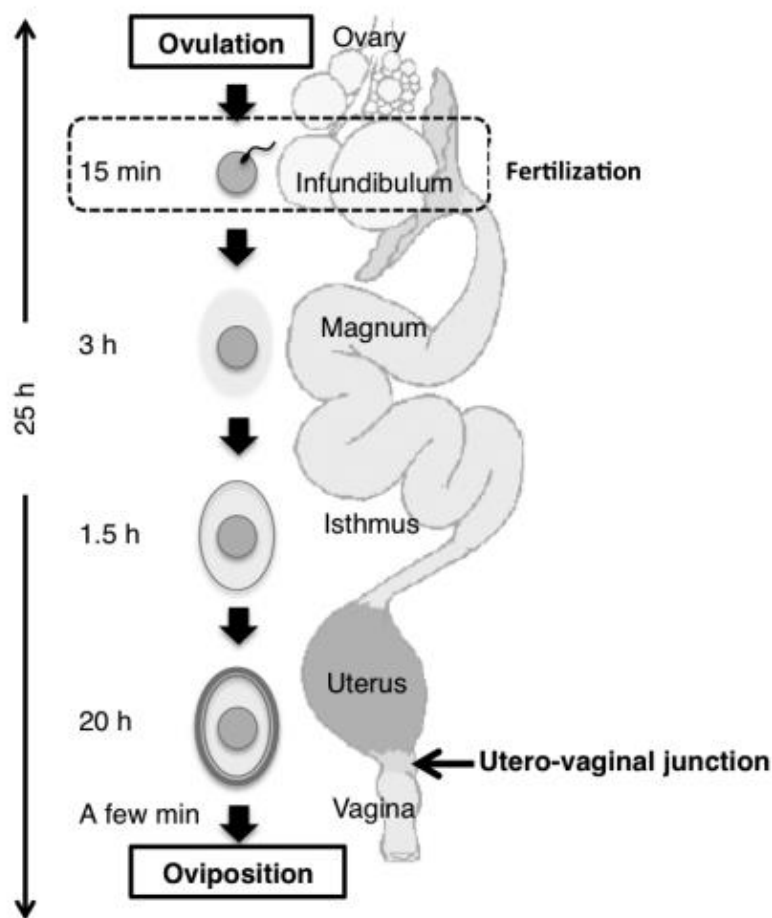


Figure 4: Diagram of a hen reproductive tract (From Sasanami *et al.* 2013): Female avian reproductive tract consists in an ovary and an oviduct divided into six segments: infundibulum, magnum, isthmus, uterus and vagina. The ovary releases an oocyte in the infundibulum when ovulation occurs, and the fertilization takes place within 15 minutes after ovulation. The egg passes through the magnum for 3 hours where albumen is added around the egg yolk. The egg then transits for 1.5 hours inside the isthmus where egg membranes are formed. Then the calcification of the shell is done in the uterus for about 20 hours. Finally, oviposition occurs 25 hours from ovulation time, the egg passes into the vagina and is expelled through the cloaca.

3.2 Folliculogenesis

Folliculogenesis is the process of follicular growth from a stage of primordial follicle to pre-ovulatory one. The avian ovary is organised with a hierarchy of follicles at different developmental stages (Fig. 5). Primordial follicles grow into the primary follicles stage with the formation of theca interna cells. Growth continues to the pre-hierarchical follicle stage, external cells of the theca develop, and white yolk lipoprotein starts to accumulate. These follicles are then selected into hierarchical or pre-ovulatory follicles, granulosa cells become squamous to facilitate the accumulation of yolk (vitellogenesis). At this stage, the follicles grow rapidly, and the largest follicle ovulated will be captured by the infundibulum during ovulation (Hrabia 2022).

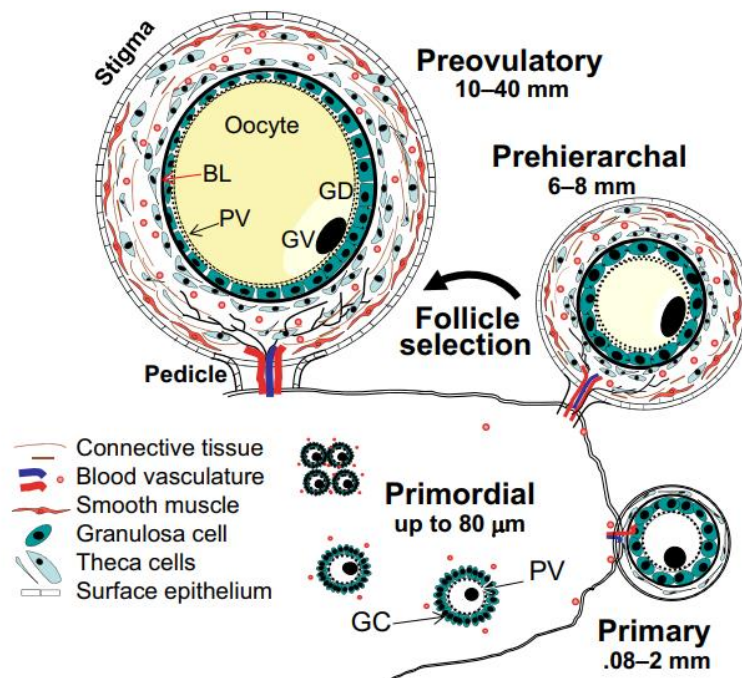


Figure 5: Schematic representation of follicular hierarchy and growth in the hen ovary (From Johnson 2015): germinal vesicle (GV), perivitelline membrane (PV), granulosa cells (GC), basal lamina (BL)

3.3 Spermatogenesis

During spermatogenesis, which occurs in the seminiferous tubules of the testis, diploid cells are transformed into haploid cells by a complex process involving mitotic and meiotic divisions and cellular differentiation leading to the formation of male gametes, the spermatozoa (Fig. 6). Primary spermatocytes enter the first meiosis division and become secondary spermatocytes that undergo a



second meiosis division to obtain round spermatids (Lin & Jones 1993). The full process of spermatogenesis has been mainly described in the Japanese Quail and in the Domestic Fowl. Four types of spermatogonia cells have been described in Japanese quails based on cell morphology, nuclear size, chromatin appearance, and their topographic position: Dark spermatogonia (Ad) are stem cells and each division of one of these cells will result in two daughter cells, one Ad, maintaining the stock of stem cells and one pale spermatogonia (Ap1); from Ap1 mitotic divisions a pale spermatogonia 2 (Ap2) will derive, and from the Ap2 mitosis a spermatogonia B (Jones & Lin 1993). Spermatogonia cells B undergo mitotic division and differentiate into primary spermatocytes. These cells are the largest germ cells in the testes and eight phases based on the chromosome's structures have been described in the Japanese quail: preleptotene, leptotene, zygotene, pachytene, diplotene, diakinesis, metaphase, and anaphase (Lin *et al.* 1990) (Fig. 7). The last step of spermatogenesis, spermiogenesis, will differentiate round spermatids into sperm cells (Hess & Renato de Franca 2008). The number of morphological steps to achieve this process depends on the species. In the quail (Lin and Jones, 1993; Lin *et al.*, 1990) and in the Turkey (Aire 2003) 12 steps have been identified (Fig. 8), whereas in chickens eight to 10 steps have been reported and only six in the House Sparrow (Góes & Dolder 2002). During spermiogenesis the acrosome and axoneme will be formed, a part of the cytoplasm is removed, and nuclear condensation will occur with a replacement of histones by protamines (Vizcarra *et al.* 2022 ; Fig. 8). Once spermiogenesis is completed, spermatozoa are released into the epididymis, this is referred as spermiation (Asano & Tajima 2017). The duration of spermatogenesis corresponds to the time between the division of a spermatogonia and spermiation (Asano & Tajima 2017). The duration of spermatogenesis varies among species, it lasts 14 days in the chicken (de Reviers 1968), 9.5 to 11 days in the Japanese Quail (Amir *et al.* 1973; Clulow & Jones 1982) and 12 days in the Barbary Drake (Marchand *et al.* 1977; Marchand 1979). Avian spermatogenesis is much shorter than mammalian spermatogenesis which ranges from 34 days in the boar to 74 days in man (Roosen-Runge 1977).

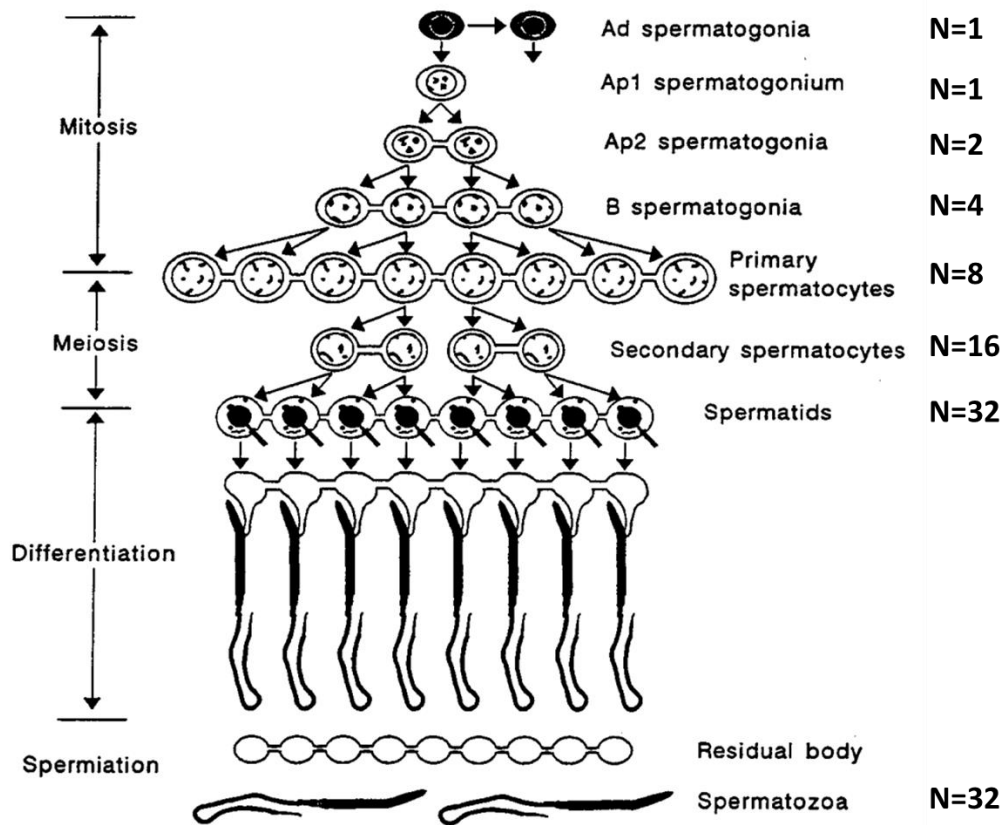


Figure 6: Representation of the spermatogenesis process in the Japanese quail (modified from Jones and Lin, 1993): Spermatogenesis involves three major phases : (1) the spermatogonial stage which involves renewal of stem spermatogonia Ad and proliferation by two or more mitotic divisions of one of the products (Ad, Ap1, Ap2 and B) of the renewal division; (2) meiosis: the spermatocyte stage which involves two reduction divisions (primary and secondary spermatocytes); and (3) spermiogenesis, the spermatid stage which involves differentiation to produce spermatozoa. Each Ad spermatogonia division will result in one Ad, one Ap1, 2 Ap2, 4 B spermatogonia, 8 primary spermatocytes, 12 secondary spermatocytes, and 32 spermatids will differentiate into 32 spermatozoa.

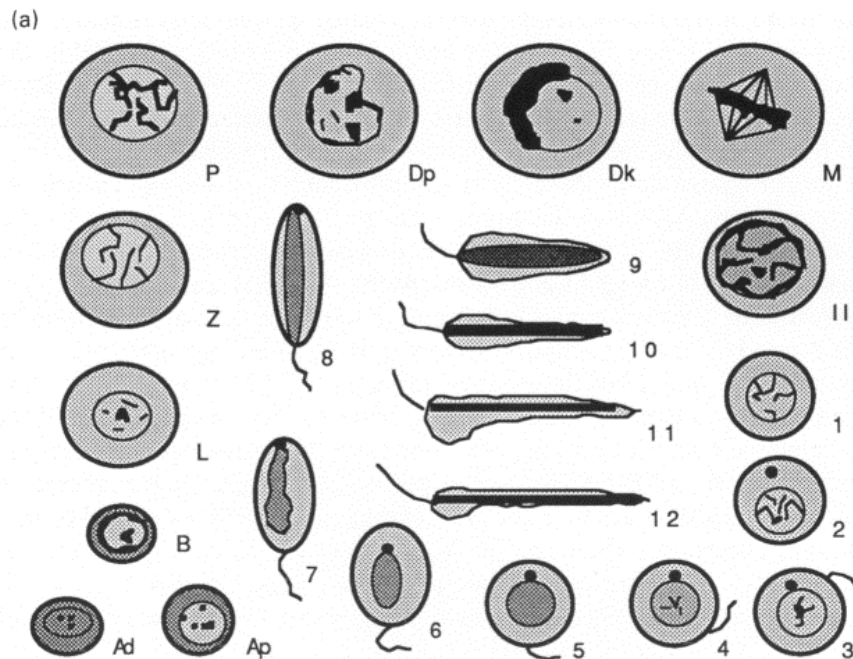


Figure 7: Schematic representation of the spermatogenesis steps (From Lin *et al.* 1990): Ad, dark type A spermatogonia; Ap, pale type A spermatogonia; B, type spermatogonia; L, leptotene primary spermatocytes; Z, zygotene primary spermatocytes; P, pachytene primary spermatocytes; Dp, diplotene primary spermatocytes; Dk, diakinesis of primary spermatocytes; M, Metaphase primary spermatocytes; An, anaphase primary spermatocytes; 1-12, Step 1-Step 12 spermatids.

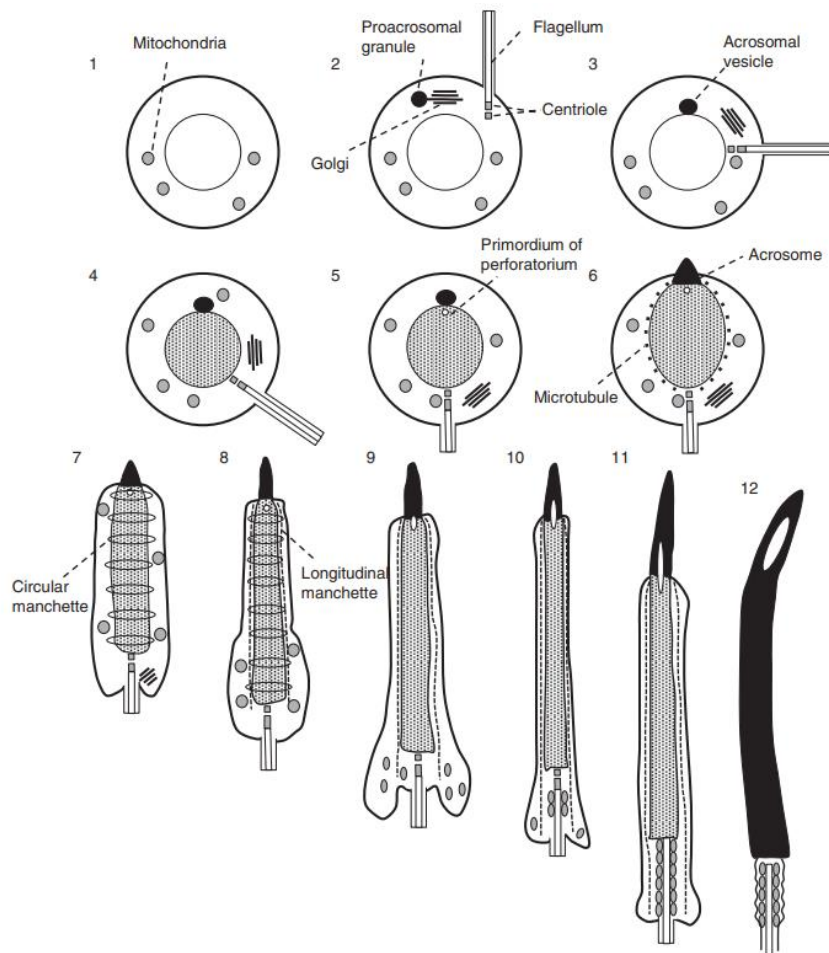


Figure 8: Schematic diagram of spermiogenesis steps in the Japanese quail (From Jones & Lin 1993; Asano & Tajima 2017): Spermiogenesis consists in 12 steps divided in 4 phase: (1-3) Golgi phase, (4-6) cap phase, (7-9) acrosome phase, (10-12) maturation phase. During the Golgi phase (1-3), the round nucleus of spermatids contains scattered chromatin aggregation and chromatin condensation will start. Multiple proacrosomal granules of the Golgi complex will fuse to form a large acrosomal vesicle that will be juxtaposed to the nucleus. The centriolar complex made of proximal and distal centrioles appears close to the acrosome in development and become a flagellum. During the cap phase (4-6), the round nucleus continues his condensation and starts its elongation. The nuclear membrane at the contact point of the acrosome vesicle and nucleus invaginates into the nucleoplasm forming the endo-nuclear canal where the *perforatorium* will be formed later. The manchette is a microtubular structure consisting in a circular manchette (CM) and longitudinal manchette (LM). During the acrosome phase (7-9), the nucleus shape changes and become thinner and more elongated. CM and LM are positioned along the external surface of the nucleus. A fibrous sheath surrounds the flagellum, and mitochondria elongates and will then align along the flagellum. During the maturation phase (10-12), the nucleus continues his elongation, the acrosome elongates and forms a cap-like structure over the tip of the



nucleus and the *perforatorium* extends from the base of the endo-nuclear canal to the tip of the acrosome. Elongated mitochondria are aligned with the midpiece and form a helical sheath. Manchettes disassemble which allows the mitochondria apposition to proximal axons. Mature spermatids are located at the luminal surface of seminiferous epithelium while being held by a slip of Sertoli cell cytoplasm. Most avian spermatozoa upon spermiation do not present a cytoplasmic droplet except in emus (Du Plessis & Soley 2014) and domestic ducks (Simões *et al.* 2012).

3.4 Neuroendocrine control of reproduction

3.4.1 The Hypothalamus-pituitary-gonadal axis

Neuroendocrine system and its different factors have mainly been studied in domestic species (chicken and Japanese quail) and some passerines such as zebra finches and starlings (Scanes 2022). Neuroendocrine system controls reproduction via the hypothalamic-pituitary-gonadal (HPG) axis. Gonadotropins releasing hormone (GnRH) secreted in the hypothalamus modulates the release of gonadotropins hormones, follicle-stimulating hormone (FSH), and luteinizing hormone (LH), by the pituitary gland that will stimulate the production of sexual steroids by the gonad leading to the production of gametes and the expression of secondary sexual traits and reproductive behaviours (Leska & Dusza 2007). This system is controlled by environmental factors but also by internal factors via feedback of sex steroids on the hypothalamus and pituitary gland (see Fig. 9).

Two types of gonadotropins releasing hormone (GnRH) have been identified in birds: GnRH1 and GnRH2 (Dunn *et al.* 1993; Ikemoto & Park 2006). GnRH1 structure seems conserved across birds as it is identical in all avian species where it has been documented (in chickens, geese, zebra finches and the starlings) (Scanes 2022). GnRH1 is expressed in cell bodies in the preoptic area of the hypothalamus and released by neurosecretory terminals in the median eminence (Stevenson *et al.* 2009). Both GnRH1 and GnRH2 stimulate the release of LH and FSH *in vivo* and *in vitro* in Japanese quails, although the action on LH is more marked (Hattori *et al.* 1986). GnRH2 is not detected in the median eminence of chicken and at puberty hypothalamic levels of GnRH1 rises whereas levels of GnRH2 do not change (Sharp *et al.* 1990). Therefore, GnRH1 is believed to be the main releasing factor of LH (Scanes 2022). GnRH2 is likely to be involved in reproductive behaviours as in white-crowned sparrows, (*Zonotrichia leucophrys*) brain injection of GnRH2, but not GnRH1, increased female courtship behaviour (Maney *et al.* 1997). The release of GnRH1 is stimulated and inhibited by several neurotransmitters and neuropeptides such as norepinephrine and neuropeptide Y that stimulate GnRH1 release whereas dopamine, β endorphin and Gonadotropin inhibitory hormone (GnIH) are inhibitors. GnIH cell number varies through reproductive cycle with a peak at the beginning of incubation and besides its inhibitor action on GnRH, GnIH also decreases the release of FSH and LH (Scanes 2022).

GnRH is released in the median eminence and stimulates LH and FSH production via binding to GnRH receptors (Joseph *et al.* 2009). Two GnRH receptors subtypes, type I (Sun *et al.* 2001) and type III (Shimizu & Bédécarrats 2006) have been identified in chickens and named according to Millar *et al.* (2004) classification. Both subtypes belong to the G protein coupled receptors (GPCR) family and are expressed in the anterior pituitary gland, where they are co-located with FSH and LH (Joseph *et al.* 2009). The two gonadotropins, LH and FSH are glycoproteins both consisting of two subunits, α -subunit common to both and a specific β -subunit, necessary for biological activity (Kobayashi & Ishii 2002). Their receptors' (LHR and FSHR) structures have been identified in poultry and wild species and are expressed in ovarian and testicular cells. FSH and LH regulate gonad's activity by binding to their receptors, activating several signalling pathways that will lead to steroidogenesis and cell proliferation necessary for gametes production (Scanes 2022).

An inhibitor effect of GnIH on gonadotropin secretion in response to unfavourable environmental condition is also suggested.

3.4.2 Control of gametogenesis by the neuroendocrine system

Folliculogenesis and spermatogenesis are both controlled by the HPG through gonadotropins and gonadal steroids stimulation and inhibition. In females, LH and FSH stimulates sex steroids synthesis by follicular cells through binding to their receptors, LHR and FSHR (Hrabia 2022). Steroidogenesis in the ovary is mediated by several follicular cells: granulosa cells, thecal interstitial cells and aromatase cells of the theca externa. Sex steroid synthesis is initiated with cholesterol transport from outer to inner mitochondrial membrane by StAR protein. Cholesterol is then converted into pregnenolone by CYP11A1 enzyme in the inner mitochondrial membrane (Hrabia 2022). From this step, two different pathway $\Delta 4$ and $\Delta 5$ can lead to sex steroid production. Pregnenolone is converted to progesterone and then to androstenedione through $\Delta 4$ pathway. Whereas in $\Delta 5$ pathway, pregnenolone is first converted to dehydroepiandrosterone (DHEA) and then to androstenedione. Both pathways occur in granulosa and theca cells, however granulosa cells preferentially produce sex steroids through $\Delta 4$ pathway whereas $\Delta 5$ pathway is preferred by theca cells (Lee *et al.* 1998). Androstenedione is converted to testosterone that is aromatized to oestradiol (Hrabia 2022). Expression of StAR protein in granulosa cells increases during follicular growth and is more elevated in the largest pre-ovulatory follicle (Sechman *et al.* 2011; Sechman *et al.* 2014).

In chicken hens ovary, pre-hierarchical follicles are the main source of oestrogen whereas preovulatory follicles are mainly responsible of progesterone production (Lee *et al.* 1998). This can be explained by a different expression of FSHR and LHR depending on follicular growth status. FSHR can be found in theca and granulosa cells at all developmental stages but is more abundant during follicle recruitment and in preovulatory follicles and is the lowest in atretic follicles (You *et al.* 1996; Zhang *et al.* 1997). LHR is expressed in theca cells of pre-hierarchical and pre-ovulatory follicles whereas LHR is only found in granulosa cells after follicle selection. Therefore, pre-hierarchical follicle steroid synthesis stimulated by LH is attributed to theca cells (Sechman *et al.* 2016; Sechman *et al.* 2020). During follicular maturation of pre ovulatory follicles, LHR expression decreased in theca cells while it increases in granulosa cells (Zhang *et al.* 1997). Thus, LH stimulates progesterone production by granulosa cells in pre-ovulatory follicles (Hrabia 2022). LH also stimulates follicular development by activating granulosa cells proliferation of pre-ovulatory large follicles (F1-F3). FSH controls the selection of small follicles and the development of granulosa cells resulting in an increase large follicles number and progesterone production by granulosa cells (Hrabia 2022). The main action of LH in females is to induce ovulation. The pre-ovulatory LH surge occurs four to six hours before ovulation, induced by positive feedback of



progesterone, provokes a sequence of modifications in the most mature pre-ovulatory follicle (F1). First the breakdown of the germinal vesicle occurs, then the junctions between the granulosa cells and the oocyte surface dissociate, after that the perivitelline space is developed and finally the meiosis resumes and will arrest in meiosis II until fertilization (Johnson 2015).

In males, gonadotropins receptors are present in Leydig and Sertoli cells (Scanes 2022). FSH acts on Sertoli cells and is an important regulator of their proliferation and differentiation. This regulation is important as sperm number produced is associated with the number of Sertoli cells present (Sharpe 1994). LH activates receptors in Leydig cells via increase of a second messenger, cAMP, initiating the steroidogenesis of androgens, mainly testosterone and androstenedione, precursor of testosterone. Testosterone is secreted in pulses that closely follow LH pulses, and its concentration in the testis are much higher than in the general circulation. Testosterone is mandatory for spermatogenetic activity, the maintenance of excurrent ducts and secondary sexual traits and to control GnRH secretion pattern. In both male and female, sex steroid modulates LH and FSH release via action on the hypothalamus or pituitary gland (Scanes 2022). This was demonstrated by gonadectomy experiments. Indeed, in the Japanese quail, gonadectomy led to increased secretion of LH and FSH (Davies *et al.* 1980). In ovariectomized domestic hens, progesterone or oestradiol decreased LH plasma concentrations of LH (Wilson & Sharp 1976b, 1976a). Androgens inhibits LH release via androgens and oestrogens receptors (Davies *et al.* 1980; Wilson *et al.* 1983; Fennell *et al.* 1990). In females, both oestradiol and progesterone act as negative feedback on LH production. However, depending on the cycle stage, progesterone can also act as positive feedback as progesterone induces the pre-ovulatory surge of LH.

3.4.3 Control of seasonal breeding

Seasonal birds breed at specific time of the year for their chicks to hatch in favourable environmental conditions with food availability. Multiple factors can regulate seasonal breeding. Photoperiod or daylength is a good indicator of the most favourable time to breed especially in temperate zones (Scanes 2022). Indeed, LH and FSH secretion increases with increasing daylength in temperate zone birds such as Japanese quails (Follett & Maung 1978; Henare *et al.* 2011) and starlings (Dawson *et al.* 1985). Reproduction and sexual development is induced by photoperiod in poultry in the same way (Onagbesan *et al.* 2006). Light is detected in the hypothalamus by a photopigment called rhodopsin (Leska & Dusza 2007). Rhodopsin transforms light photon energy in neural impulses and light induces rhodopsin degradation into opsin, located in the medio basal hypothalamus (MBH). Opsin neurons form synapses on GnRH neurons and the information transfer occurs through specialized glial cells called tanycytes (Leska & Dusza 2007). Activation of photo receptor by long days modulates



tanocytes' transcription of two enzyme genes: 2 iodothyronine deiodinase (DIO2; (Yoshimura *et al.* 2003)) is up-regulated and 3 iodothyronine deiodinase (DIO3; (Yasuo *et al.* 2005)) is down regulated. DIO2 converts prohormone thyroxine (T4) to bioactive triiodothyronine (T3) and DIO3 transforms both T3 and T4 into inactive metabolites (Yasuo *et al.* 2005). Thus long photoperiod results in a 10 fold increase of T3 in the brain, via thyroid hormone receptors on glial cells, stimulates GnRH releases (Yoshimura *et al.* 2003; Yamamura *et al.* 2004). In contrast, during short days, the pattern of DIO2 and DIO3 expression suppresses the high level of T3, and GnRH releases is inhibited (Yasuo *et al.* 2005).

Temperature and weather conditions are also involved in reproduction timing. The induction of reproduction is accelerated by high temperature (Silverin *et al.* 2008; Schaper *et al.* 2012) and slowed down by low temperatures (Wingfield *et al.* 2003; Visser *et al.* 2009) in passerine birds. However, very high temperatures can reduce reproductive success (Cruz-McDonnell & Wolf 2016). Rainfalls control reproduction timing of birds in arid-regions and usually the first rainfall induces the onset of reproduction, this is the case for example in blue cranes (Altwegg & Anderson 2009), sociable weavers (Mares *et al.* 2017) and zebra finches (Zann *et al.* 1995). Water availability can also influence reproduction timing in song sparrows (*Melospiza melodia*), where it increases photoperiodic responses (Wingfield *et al.* 2012). Food availability can also control reproduction as gonadotropin release can be modulate by nutritional status. Food restriction decreases LH concentrations in Japanese quails (Kobayashi & Ishii 2002), LH and FSH concentration in chickens (Bruggeman *et al.* 1998).

Prolactin (PRL) is involved in the control of reproductive behaviour related to incubation and care of young, called broodiness (Scanes 2022). In chickens (Riddle *et al.* 1935) and turkeys (Youngren *et al.* 1991), PRL induces broodiness, and a study has shown that turkey hens PRL-immunized did not show incubation behaviour (Crisóstomo *et al.* 1998). PRL concentration increases during incubation and declines during broodiness (Smiley & Adkins-Regan 2016).

Thyroid hormone as a dual role in the regulation of seasonal gonadal development and regression. Under long day conditions, T3 stimulates GnRH secretion and activates the HPG axis. In contrast, short day conditions and low temperature act together to inhibit GnRH secretion and shut down the HPG axis. Low temperature leads to higher food consumption resulting in increased expression of DIO2 in the liver. Circulating T3 acts on the testis to regulate T3 responsive genes involved in regression and in promoting apoptosis of Sertoli and Leydig cells (Young *et al.* 2001; Ikegami *et al.* 2015). The degenerated Leydig cells become fibroblast-like structures and will be transformed into a new generation of juvenile interstitial cells during the next breeding season (Mori & George 1978). Sertoli cells proliferation will stimulate FSH once the HPG is re-activated. T3 also acts on the ovary and

induces follicular atresia of hierarchical follicles by apoptotic cell death pathway. Apoptosis starts in the granulosa cells and then continues in theca cells (Hrabia 2022).

3.5 Avian sperm

3.5.1 Morphology and physiology

To be able to reach and fertilize an egg, sperm are specialized cells. Similarly to mammalian sperm, avian sperm consists of four distinct compartments: the acrosome, the nucleus, the midpiece, and the flagellum. The acrosome, positioned on top of the nucleus, is a vesicle containing proteolytic enzymes necessary to digest the perivitelline layer (IPVL). The nucleus shape is long and thin compared to the mammalian sperm, thus also contains condensed chromatin. The midpiece presents a variable number of mitochondria, producing the energy for flagellar movement (Asano & Tajima 2017). Avian sperm morphology can be divided into two main types: passerine and non-passerine types (Fig. 10). The latter is the simplest and is characterized by a long and filiform nucleus with a short acrosome. Passerine sperm morphology is more complex, with longer acrosome and a helicoidal nucleus. In some passerine birds, the acrosome length can even exceed the length of the nucleus (Jamieson 2007).

Avian sperm morphometry is highly diverse across species with a total sperm length range from 42.7 μm in the Red-backed shrike (*Lanius collurio*) (Briskie *et al.* 1997) to 292.4 μm in the Reed bunting (*Emberiza schoeniclus*) (Dixon & Birkhead 1997). The number of mitochondria contained inside the midpiece is also highly variable, ranging from 6-12 in the White Pekin duck (*Anas platyrhynchos*) (Majhi *et al.* 2016) and up to 1400 in the Japanese quail (Korn *et al.* 2000). Sperm length has been suggested to contribute to sperm competition as it has been found to be positively correlated with sperm velocity. Lüpold *et al.* (2009) reported positive correlation of midpiece and flagellum length with sperm velocity in 40 passerine species. Similarly, in zebra finches, sperm length and velocity are both phenotypically and genetically correlated (Mossman *et al.* 2009; Bennison *et al.* 2016) and longer sperm fertilize more eggs (Bennison *et al.* 2015). In turkeys, high motility was correlated with sperm head length (Manier *et al.* 2019). However, negative correlations have also been reported. In five populations of Canary Islands chiffchaff (*Phylloscopus canariensis*) (Cramer *et al.* 2021), and in Spanish house sparrows (Cramer *et al.* 2015), sperm length and velocity were negatively correlated.

Energy supply is necessary for sperm to swim and fertilize an egg; sperm metabolism has mainly been studied in mammalian sperm. Adenosine triphosphate (ATP) is the source of energy of sperm cells and can be produced by two metabolic pathways: glycolysis and oxidative phosphorylation. These processes take place in different compartments: in mammalian sperm, glycolysis occurs in the head and in the fibrous sheath of principal piece of the flagellum whereas oxidative phosphorylation occurs



in the mitochondria of the midpiece (Du Plessis *et al.* 2015). Production of ATP is more efficient with oxidative phosphorylation as it produces 30 molecules of ATP for one molecule of glucose whereas glycolysis produces only two (Du Plessis *et al.* 2015). In mammals, oxidative phosphorylation is believed to be the main source of ATP production but glycolysis is still needed (Du Plessis *et al.* 2015; Davila *et al.* 2016). In birds, sperm energetic metabolism is still poorly understood and literature scarce. In chicken spermatozoa, oxidative phosphorylation has been suggested to be the main source of ATP production (Froman & Feltmann 1998; Setiawan *et al.* 2020). However, in the study of Setiawan *et al.* (2020), when mitochondrial oxidative phosphorylation was inhibited, sperm motility decreased, but still 60% of sperm were motile suggesting that flagellar motility is also supported by glycolysis. Moreover, they showed that ATP in the midpiece is produced via both glycolysis and oxidative phosphorylation, and enzymes necessary for glycolysis were detected in the midpiece and at the principal piece of the flagellum. In a passerine bird, the Zebra Finch, sperm length is positively correlated with velocity up to a certain limit as velocity decline was observed in very long sperm (Bennison *et al.* 2016). Surprisingly, the ATP content of mitochondria was negatively correlated the midpiece length, meaning longer midpieces contained less ATP (Bennison *et al.* 2016). These results might suggest an adaptative metabolic pathway in different sperm compartments in relation to mitochondrial length, velocity of sperm with long midpiece might be supported by glycolysis in the principal piece of the flagellum, for example. Also, the ATP content might not be linked only to motility observed shortly after insemination but also associated to sperm longevity and metabolism requirement inside the female tract during storage.

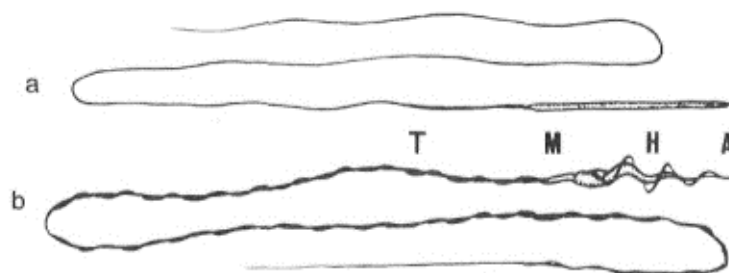


Figure 10: Drawings of the two types of avian sperm morphology (King & McLelland 1984): a) Non-passerine or Galliformes type; b) Passerine type; A: acrosome, H: head, M: midpiece, T: tail or principal piece of the flagellum

The ejaculate consists in two components, a cellular one, sperm cells, and a fluid part, the seminal plasma secreted by male reproductive tract. Composition of avian seminal plasma has been studied in poultry and the main components are inorganic ions, sugars, proteins, peptides and hormones (Druart & Graaf 2018; Santiago-Moreno & Blesbois 2020). These different components



interact with spermatozoa and are involved in sperm metabolism, sperm transit, sperm storage and fertilization (Santiago-Moreno & Blesbois 2020). Differences in seminal plasma composition have been associated with sperm quality and fertility. For example, turkey males suffering from yellow semen syndrome have reduced sperm quality and fertility and their seminal plasma presents a different composition compared to healthy males with a differential expression of 49 proteins and levels of 1549 metabolites (Słowińska *et al.* 2015; Slowinska *et al.* 2018). Different proteomes have also been associated with sperm motility (Li *et al.* 2020). Recently, the presence of extracellular vesicles have been reported in chicken seminal plasma (Cordeiro *et al.* 2021; Luo *et al.* 2022; Han *et al.* 2023), and a differential size and protein profiles of these extracellular vesicles in seminal plasma of fertile and sub-fertile chickens (Cordeiro *et al.* 2021).

3.5.2 Sperm storage

a) In the male tract

After production in the testis, sperm cells transit and are stored in the epididymis and the vas deferent. In mammals, a sperm maturation process occurs in the epididymis and is mandatory to gain motility and fertilization ability (James *et al.* 2020). Due to the much smaller epididymis in birds compared to mammals, it was long believed that avian sperm maturation only happened in the vas deferens, and that this maturation was mainly an acquisition of motility (Munro 1938; Howarth 1983).

However, several studies showed evidence that fertilization capacity changes additionally to motility and not only in the vas deferens. Ahammad *et al.* (2011a, 2011b) demonstrated a gradual maturation process with an increase of motility, viability, acrosomal proteolytic activity, and penetrating ability through transit in the fowl genital tract, from the epididymis to distal vas deferens. In the Japanese quail, sperm motility increases in the epididymis (Nixon *et al.* 2014). González-Santos *et al.* (2019) showed changes in acrosomal reaction capacity and sperm morphology in the fowl. More recently, in the rooster, an increase of sperm chromatin compaction during transit along vas deferens was observed (Bernal *et al.* 2022). Taken together, these studies provide evidence that epididymal and vas deferent transit may also be important for avian sperm to acquire full fertilizing capacity.

b) In the female oviduct

After copulation spermatozoa are stored inside the oviduct inside specialized structures called sperm storage tubules (SSTs) (Blesbois & Brillard 2007) and only one per cent of sperm inseminated reach the SSTs (Bakst 1994). The main site of storage where SSTs can be found is located in the utero vaginal junction and in some species a secondary storage is located in the infundibulum. The time of



storage can range from a few days in the Dove (*Streptopelia risoria*) (Zenone *et al.* 1979), and up to 72 days in the Turkey (Lorenz 1950). The abundance of SSTs is also variable across species; only a few hundred are found in passerine birds (Briskie 1996) whereas turkey hens can have as much as 20,000 (Goodrich-Smith & Marquez 1978). During this storage time, sperm motility is inactivated, and sperm metabolism thought to be in a quiescent state (Matsuzaki & Sasanami 2022). Froman (2003) has suggested a rheotaxis mechanism in chickens, sperm would stay motile inside SSTs and swim against fluid current generated through protein channels of SST epithelial cells. Sperm would be released when their velocity drop upon a threshold allowing retrograde movement. The presence of aquaporine channels in SSTs epithelium revealed by immunochemistry (Zaniboni & Bakst 2004) is consistent with Froman (2003) hypothesis. More recently, rheotaxis was reevaluated in chickens with an *in vitro* experiment (El-Sherry *et al.* 2022). They used a microfluidic channel generating a current to mimic SST and observed formation of sperm bundles swimming against the flow and these bundles were adhering to the chamber walls. However, this may not reflect what is happening in the oviductal microenvironment and several *in vivo* investigations reported that sperm remain immotile while stored inside SSTs (Matsuzaki & Sasanami 2022). Little is known about the complete mechanisms of motility inactivation/re-activation and storage/release control before fertilization.

However, some investigations have reported several factors involved in these processes. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) contained in cloacal gland secretion (CGS), which is a major component of seminal plasma in quails, influence SST filling, $PGF_{2\alpha}$ in the CGS induced vaginal contractions and opening of the entrance of SSTs. Intravaginal injection of $PGF_{2\alpha}$ and CGS before AI with washed sperm increased sperm storage inside SST (Sasanami *et al.* 2015). $PGF_{2\alpha}$ are also present in turkeys and roosters semen (Hertelendy 1974; Kennedy *et al.* 2003).

Flagellar movement of sperm is produced by the sliding of microtubules with motor dynein ATPase (Inaba 2007). In Japanese quails, the luminal environment of SST is hypoxic and acidic due to high concentration of lactic acid (Matsuzaki *et al.* 2015). In these conditions, sperm cytosolic pH drops at 5.4 and inhibits ATPase activity of dynein. Sperm mitochondrial activity is also decreased by hypoxia (Matsuzaki *et al.* 2015). Temperature can also influence sperm motility inactivation. At 40°C (body temperature) and without Ca^{2+} , sperm were immotile and motility was restored by decreasing the temperature at 30°C in chickens, turkeys and drakes semen but not in quails (Ashizawa & Nishiyama 1978; Wishart & Wilson 1999). However, motility can be restored at 40°C by adding Ca^{2+} (Ashizawa *et al.* 1989). Acidic pH conditions at 40°C may also inactivate motility as motile sperm were observed at body temperature by increase of intracellular pH of sperm (Ashizawa *et al.* 1994). Sperm binding to SST luminal epithelial cells through a ligand receptor mechanism has been suggested (Ashizawa & Nishiyama 1983; Ahammad *et al.* 2011c). However other studies have reported stored sperm to form



a bundle resulting in the inability of all sperm to make physical contact with epithelial cells (Bakst 1983; Sasanami *et al.* 2013). Mendonca *et al.* (2019) suggested another mechanism of sperm maintenance in SSTs by studying the 3D structure of zebra finches. They showed that the entrance of SSTs is a narrow opening 3 μ m whereas the lumen diameter is 3-fold wider. They suggested SSTs to be gate-like structures, with valve-like opening only allowing only unidirectional movement and preventing sperm stored inside to be flushed out.

Sperm release from SSTs is likely to be under ovulatory cycle control and progesterone, peaking four to six hours prior to ovulation. A progesterone injection induced sperm release of almost all sperm stored within one hour post injection (Ito *et al.* 2011). Another factor has been shown to play a role in Japanese quails, in vitro experiment has shown expression and localisation of HSP70 in surface epithelium cells of UVJ, its binding to sperm through interaction with voltage dependent anion channel protein 2 (VDAC2) and activation of sperm flagellar movement (Hiyama *et al.* 2014). Additionally, treatment with anti-HSP70 antibody drastically reduced fertility (60% decline) but did not impact sperm storage inside SSTs. Therefore, HSP70 is probably involved in sperm migration toward fertilization site. Sperm storage is involved in sperm competition and in LMP (see section 1.4.2.b-c) on sperm competition). Differences in sperm quality may lead to differential sperm storage in SSTs and bias fertilization outcome. Direct evidence of such process is scarce, however sperm mix with same number but different sperm length or sperm motility biased SST filling and paternity toward sperm with longer midpiece and flagellum (Matsuzaki *et al.* 2021) and more motile sperm (Matsuzaki & Sasanami 2022).

3.6 Fertilization

Fertilization occurs in the infundibulum within 15 minutes after ovulation (Nishio & Matsuda 2017). In mammals, ovulated oocytes are surrounded by the zona pellucida and cumulus cells. However, during ovulation in birds, the granulosa cell layer surrounding the perivitelline layer (PVL) is detached and the egg is released from the follicular capsule. The ovulated oocyte is filled with yolk and surrounded by a perivitelline membrane (oolemma) and the PVL, an extracellular matrix made of fibers and zona pellucida (ZP) glycoproteins. These ZP glycoproteins are classified into six subfamilies based on phylogenetic analysis which are ZP1, ZP2/ZPA, ZP3/ ZPC, ZP4/ZPB, ZPD, and ZPAX (Spargo & Hope 2003; Goudet *et al.* 2008). These glycoproteins are highly conserved in birds and ZP1, ZP3 and ZPD are the most abundant molecules of the PVL. ZP proteins are secreted by granulosa cells of the ovary, except for ZP1 secreted by the liver (Nishio *et al.* 2014; Nishio *et al.* 2018). These glycoproteins are mandatory for fertilization as they interact with sperm and initiate the acrosome reaction (AR) (Okumura 2017; Nishio *et al.* 2018).

3.6.1 Egg-sperm interaction and acrosome reaction

The first step of fertilization is the sperm binding to the inner perivitelline layer (IPVL) at the germinal disk location. This binding induces the acrosome reaction provoking the release of the acrosome content and proteolytic enzymes that will digest the matrix and make holes in the IPVL (Stepinska & Bakst 2007). One of these proteolytic enzymes, called acrosin, has been identified and isolated in chickens (Ho & Meizel 1970; Froman 1990) and turkey sperm (Richardson *et al.* 1988). The acrosin, is a trypsin like protease, and was shown to provoke the hydrolysis of the IPVL of chicken ova *in vitro* (Ho & Meizel 1970). The presence of trypsin inhibitors, hydrolysis of IPVL was inhibited in chickens and quails IPVL *in vitro* (Howarth & Digby 1973; Kuroki & Mori 1997; Takeuchi *et al.* 2001). Sperm proteasome might also be involved in sperm penetration, as its inhibition inhibited the holes formation in the IPVL (Sasanami *et al.* 2012).

Direct observations of sperm and PVL interactions are difficult and most of studies used PVL extracts, purified ZP of PVL or recombinant proteins to explore these interactions. Several *in vitro* experiments have shown that AR is initiated while sperm is incubated with PVL homogenate and Calcium (Ca^{2+}) play an important role in this process (Sasanami *et al.* 2007; Ahammad *et al.* 2013; Nguyen *et al.* 2016; Ichikawa *et al.* 2017; Ushiyama *et al.* 2019). ZP glycoprotein interactions are mandatory for AR, in Japanese quails AR is mediated by sperm interaction with ZP1 (Sasanami *et al.* 2007; Ichikawa *et al.* 2017) and ZP3 (Ichikawa *et al.* 2017) and in chickens ZP1 (Okumura *et al.* 2004), ZP3 (Bausek *et al.* 2004) and ZPD (Okumura *et al.* 2004) induce AR.

In mammals the AR occurs with a fusion of sperm membranes at the apical region of the acrosome and the latter is completely disintegrated. A capacitation process is also needed for mammalian sperm to undergo AR. It involves structural and biochemical changes including increase of permeability and destabilization of sperm membrane to enable multiple openings at the apical region of the acrosome (Barros *et al.* 1967; Yanagimachi & Noda 1970). However, in birds, the AR appears to be different. No capacitation is needed, and AR does not seem to involve the membrane's fusion or disruption at the apical region of the acrosome. In the fowl after inducing AR *in vitro* with solubilized IPVL and CaCl_2 , the intact removal of the acrosomal cap from the posterior acrosomal region of the sperm was observed (Ahammad *et al.* 2013). Moreover, proteolytic enzymes may be released through a single circular opening formed at the base of the acrosomal cap around the sub-acrosomal rod (Ahammad *et al.* 2013). AR also involves AMP-activated protein kinase (AMPK) pathways, and (Nguyen *et al.* 2016) showed that AMPK activation happened through calcium influx in store-operated Ca^{2+}

channels (SOCs). Another group showed that the same pathway is activated via glucose uptake through glucose transporter 3 GLUT 3 on the acrosome (Ushiyama *et al.* 2019).

3.6.2 Polyspermy and syngamy

Avian fertilization is polyspermic, multiple spermatozoa must enter the egg for successful fertilization. In chickens and turkeys, it was shown that fertilization probability increases with the number of spermatozoa entering the egg. When only 3 sperm cells entered the IPVL, the egg had a 50% probability to be fertilized, whereas when 6 or more were penetrating the IPVL, fertilization probability was 100% (Bramwell *et al.* 1995; Wishart 1997).

The site of egg-sperm interaction is located at the germinal disk where the female's pronucleus resides and several dozens of sperm can enter (up to 60 in chickens and turkeys, (Wishart 1997); see Fig.11). Soon after contact and entrance of sperm in the IPVL, an outer perivitelline layer (OPVL) is added around the IPVL and composed of proteins secreted by infundibulum epithelial cells (Bellairs *et al.* 1963). This additional OPVL is trapping sperm and preventing further entry in the IPVL. Sperm cells penetrate IPVL through small holes to reach the oolemma, the inner acrosomal membrane of sperm fuses with the vitelline membrane, and the male pronucleus is released into the ooplasm (Okamura & Nishiyama 1978; Waddington *et al.* 1998; Stepinska & Bakst 2007). The sperm nuclei will decondense to form the male pronuclei (Perry 1987). When the male pronucleus formation is almost completed, the formation of the pronucleus female starts, and the second meiotic division resumes and is completed. Two pronuclei, one male and one female, are paired at the center of the germinal disk. Several supernumerary sperm pronuclei are present in the periphery of the disk. Some of these sperm asters can start mitotic divisions but will degenerate at an early cleavage stage. DNase activity restricted to the egg cytoplasm might degrade supernumerary sperm (Stepińska & Olszańska 2003; Stepinska & Bakst 2007).

Therefore, only a single bipolar spindle forms around the male and female pronuclei followed by the fusion of nuclear material, karyogamy, and the first zygotic cleavage (Snook *et al.* 2011). The fusion of the male and female pronuclei occurs 3 to 5 hours after ovulation (Perry 1987; Waddington *et al.* 1998).

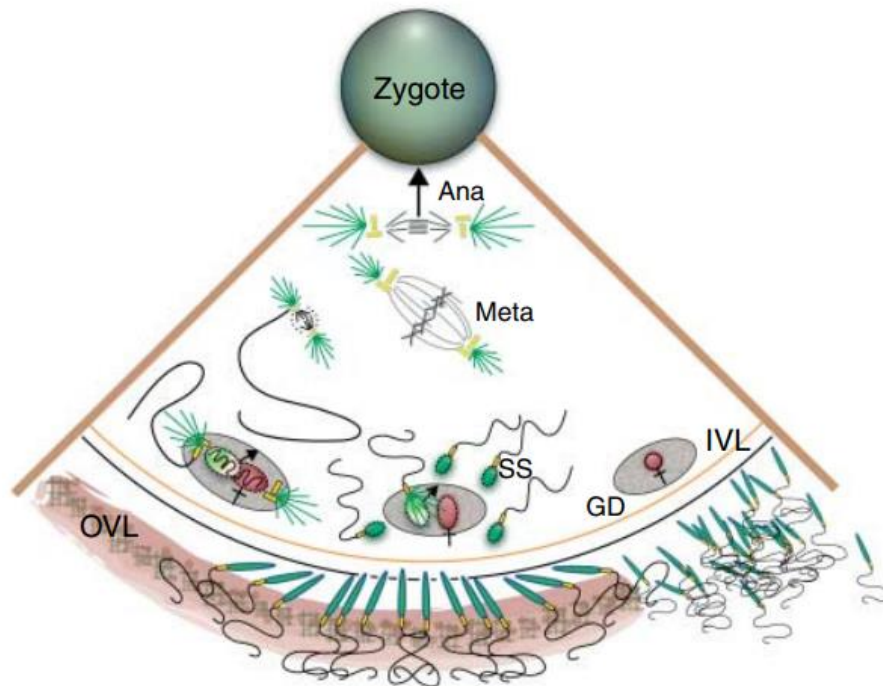


Figure 11: Schematic diagram of physiological polyspermic fertilization in birds (Snook *et al.* 2011): Fertilization events are presented in a clockwise manner on this diagram and are occurring as following: multiple sperm reach the inner vitelline layer (IVL) at the germinal disk (GD) area, where the female pronucleus resides. Shortly after sperm reach the IVL, an outer vitelline layer (OVL) is quickly assembled trapping many sperm and preventing additional sperm entry. Only one spermatozoon enters the GD and fuses with the female pronucleus (karyogamy) and the supernumerary sperm (SS) degenerate.

As shown through this review, avian reproductive biology was mainly study on poultry models and several aspects are still poorly understood.

4. THE NORTH AFRICAN HOUBARA BUSTARD

4.1 Biology and reproduction

The North African Houbara Bustard (*Chlamydotis undulata undulata*) is one of the 26 species belonging to the Otidiformes order. This resident bird lives in semi-arid steppes and its habitat ranges from North Mauritania to Egypt. It is a middle size bird measuring 55 to 65 cm of height and 125 to 150 cm of wingspan (Launay & Paillat 1990). They have a long lifespan; individuals can live more than 30 years in captivity. This bird has an omnivorous and opportunist diet due to seasonal variations of resource availability and it consists of plants, small vertebrates, and arthropods (Hingrat *et al.* 2007; Bourass *et al.* 2012).



A sexual dimorphism is observed with males being bigger (average weight of 2 kg) than females (average weight of 1.5 kg) (Fig. 12). Males also present long black and white ornamental feathers used to perform their mating display (Fig. 12). The mating system of this species corresponds to an exploded lek (Hingrat & Saint Jalme 2005a). Males aggregate in specific sites to display, with a mean distance of 550 meters between each site, and females visit leks to mate and nest outside lek sites (Hingrat *et al.* 2007). Houbara bustards are polyandrous, therefore females can mate with several males, and chicks from the same clutch can be sired by different fathers. A high proportion (60%) of multiple paternities was observed in clutches collected in the wild (Lesobre *et al.* 2010). Females can store sperm for up to 47 days (unpublished data), this period corresponds to the maximum observed delay between a single insemination and egg laying during the experiments.

The breeding season lasts from January to June, females lay one clutch of 1 to 4 eggs that they incubate for 22 to 24 days (Gaucher 1995). If a clutch is lost, females can lay a replacement clutch (Saint Jalme *et al.* 1994). After hatching, chicks are reared only by the mother and receive no parental care from males. They can fly after 1 month and fledge at around 3 months of age (Hingrat & Saint-Jalme 2005b; Hardouin *et al.* 2012). In the wild males start establishing a display site at a mean age of 2.1 years and females start laying eggs at a mean age of 1.6 years (Hardouin *et al.* 2014). In captivity, sexual maturity is reached from 1 to 2 years old for males and from 1 to 3 years old for females (Saint Jalme *et al.* 1994).

In captivity, eggs are collected on a daily basis and artificially incubated, so females will lay consecutive clutches until the end of the breeding season. On average, female breeders lay four clutches of two eggs with six to nine days of interclutch. The number of inseminations depends on the number of eggs within a clutch, for a clutch of one egg females are inseminated once whereas for clutches of two and more eggs, females are inseminated twice in sequence with a two-day interval between inseminations.



Figure 12: North African houbara bustard female (left) and male performing a display (right)

4.2 Conservation status and captive breeding program

Practiced for more than four thousand years, falconry is a very important cultural tradition in the Arabic peninsula and was registered in the UNESCO intangible cultural heritage list in 2012 (<https://ich.unesco.org/en/RL/falconry-a-living-human-heritage-01708>). Traditionally, hunting used to be seasonal, falcons were captured and trained to hunt Asian houbara bustards (*Chlamydotis macqueenii*) during their winter migration. In the late sixties, houbara hunt intensified, hunting area spread and poaching and trading of living birds to train falcons developed (Bailey et al. 1998; Tourenq et al. 2005). Added to these hunting and poaching threats, habitat loss and degradation caused a drastic and rapid decline of houbara population (Goriup 1997; Le Cuziat et al. 2005). The species (Asian and North African Houbara) are now classified as vulnerable by the international union for conservation of nature (2023).

Aiming to reinforce natural populations of houbara bustard and sustain hunting activities, conservation programs started. The first center, the National Wildlife Research Center (NWRC), was created in 1986 in Taif in Saudi Arabia. Later other centers opened and the International Fund for Houbara Conservation (IFHC) was founded in 2006 aiming to support and gather the different bustard conservation programs. The IFHC now represents three captive breeding centers, one in Morocco for North African houbara bustards, in UAE and in Kazakhstan for Asian houbara bustards.

The Emirates Center for Wildlife Propagation (ECWP) (Fig. 13) (<https://www.ecwp.org/>) was founded in 1995 by Sheikh Zayed Bin Sultan Al Nahyan in eastern Morocco aiming at reinforcing and sustaining natural populations of North African houbara bustards compatible with falconry traditions. The founder population was established with eggs collected in the wild. All chicks are produced by



artificial insemination (AI) under strict genetic management to ensure genetic diversity and evolutionary resilience within the captive flock (Rabier *et al.* 2020). Every year, some of the chicks are produced to renew the captive breeding flock and others are intended to be released. In 2023 the breeding flock of the ECWP counted 7713 breeders, 4752 females and 2961 males. Females laid more than 37,000 eggs and produced 21,870 chicks. From these, 1205 were selected to be future breeders of the captive flock and 20665 were birds intended for release or used for falcon training. From 2018 to 2022, 78 154 birds were released with an average of 15 630 birds per year for these 5 years.



Figure 13: The Emirates Center for Wildlife Propagation (ECWP) located in Missour, Morocco.

4.3 A model species for sexual selection and aging

To ensure the success of a captive breeding program, it is essential to have a thorough understanding of the biology and reproductive characteristics of the species being bred, particularly when assisted reproductive techniques, such as artificial insemination (AI), are employed. The North African Houbara Bustard has been successfully bred in captivity for over 30 years. During this time, knowledge was acquired, and procedures were developed regarding various aspects of the breeding biology of the species, including semen collection, sperm quality evaluation, cryopreservation, artificial insemination, and incubation. Houbara bustards are long-lived birds, and in the captive breeding center, males are used as breeders until an advanced age. Additionally, the large number of birds available in controlled conditions makes them a good model for studying reproductive aging in a wild species. Several studies have reported the reproductive traits of houbara and the contribution of male age to reproductive success. Saint Jalme *et al.* (1994) documented the parameters of ejaculates, including volume, concentration and sperm count, and their relationship with artificial insemination, the timing of insemination, and the hatching rate. A study was conducted on the seasonal pattern of reproductive hormone production in both male and female houbaras (Saint Jalme *et al.* 1996). Male



fertility peaks at four years of age, when a maximum number of ejaculated spermatozoa and mass motility are observed and declines after six years of age (Preston *et al.* 2011).

Annual home and breeding ranges, distribution of displaying sites, breeding behaviours and food availability were reported and houbara mating system was characterized as an exploded lek mating system (Hingrat *et al.* 2004; Hingrat & Saint Jalme 2005a; Hingrat *et al.* 2007). Male sexual display behaviour was studied both in captivity and in the wild. Visual and acoustic signals produced during courtship were analysed in wild birds and vocal signals, called booms, are characterized by low frequency allowing propagation over long distance up to 600 m (Cornec *et al.* 2014; Cornec *et al.* 2017). In captivity booming calls vary over breeding season, and low frequency signals are positively associated with display activity (Cornec *et al.* 2015). These booms are also likely to be indicator of genetic quality and identity as in captivity kinship is associated with frequency and temporal characteristics of vocalizations (Cornec *et al.* 2019).

Breeding performance in captivity and in the wild were investigated. The effect of translocation on breeding parameters was explored in free-ranging females and showed that captive bred released females are successfully able to breed in the wild (Bacon *et al.*, 2018). Moreover, age and inbreeding have negative impact on sperm traits, display behaviour, hatching probability, egg volume and weight. Over generations, motility, sperm number and activity display increases. Age, inbreeding and captivity generation number have interactive effects, showing the importance of including these parameters in the design of genetic management plans for conservation breeding programs (Rabier *et al.* 2021).

Sperm traits, reproductive success, male reproductive senescence, and post copulatory selection were also explored in captive birds. Senescence pattern in several reproductive parameters was observed in both males and females (Rabier *et al.* 2021). Sperm number, motility, number of displaying days, number of eggs laid per year, hatching success and weight decreases with age. Additionally, paternal age has been shown to negatively impact offspring, reducing hatching success and offspring growth and survival (Preston *et al.* 2015). An intergenerational effect of paternal age was also observed, with male offspring of older fathers producing less sperm (Vuarin *et al.* 2021). It is evident from these various studies that houbara males are affected by reproductive senescence, with a negative impact on their reproductive success and on the fitness of their offspring. Positive phenotypic and genetic covariation have been reported between sperm number and display rate, moreover these traits are heritable (Chargé *et al.* 2013; Chargé *et al.* 2014).

Houbara females are polyandrous and can mate with multiple males in the wild, leading to the occurrence of post-copulatory selection. Under these conditions, biased fertilization towards young males may be expected to limit the fitness cost of advanced paternal age. As competitive mating can



be mimicked with artificial insemination, houbara are a useful model species for studying post-copulatory selection. Previous research has investigated male age contribution in sperm competition. When female individuals are inseminated with a sperm mix from both old and young males, with an equal contribution of sperm number and similar motility score, fertility is biased towards young males (Vuarin *et al.* 2019a). Selection on ejaculate traits was also explored and showed that selection on motility and morphology was increased by sperm competition (Vuarin *et al.* 2019b). Female cryptic choice was also explored. To test for a possible avoidance of fertilization by a related male, females were inseminated with sperm mix of three males of different kinship level (unrelated, half sibling and cousins). Females did not select sperm from unrelated males, siring success was similar but eggs fertilized by males that were related to the females had lower hatching rate due high embryonic death (Vuarin *et al.* 2018).

The effect of enforced monoandry on female investment into reproduction was also explored by comparing reproductive investment of two lines of females, produced either by monoandrous or polyandrous inseminations over three generations. Females of polyandrous lines had higher investment as they laid more eggs and produced heavier chicks. Thus, removing the possibility of postcopulatory selection to operate can impair female reproductive investment (Sorci *et al.* 2021).

Another study has shown that the position in the mating sequence and the delay between insemination and egg-laying are the most important predictors of siring success (Sorci *et al.* 2023). In the dataset they used, it was found that 63% of eggs were fertilised by males in the last position of the mating sequence, indicating the occurrence of the last male precedence in houbara. Sperm number and mass motility index were also found to be positively associated with siring success.

However, there are still several unanswered questions. Although LMP occurs in houbara, the physiological mechanisms behind this phenomenon are not yet fully understood. To study this process, a deeper understanding of the species' reproductive biology, particularly its anatomy, is necessary. The anatomy and histology of the houbara reproductive tract have not been previously reported. Investigating these aspects would provide a better understanding of the species' biology and allow for further investigation into the storage dynamics inside the female oviduct and the possible mechanisms leading to LMP. In past studies, sperm morphology was analysed by using the eosin-nigrosin staining technique. With his technique, both sperm viability and morphology are analysed. Viable sperm cells do not absorb the stain and appear white, while sperm cells with impaired membrane integrity absorb the stain and take on a pinkish coloration (Lindsay *et al.* 1999; Chargé *et al.* 2013). Some sperm defects such as double flagella, double heads or swollen membranes were possible to classify, but these defects only partially represent the abnormalities that can be observed in houbara sperm. Sperm



morphology classification was never standardized and published for the Otidiformes order before, making it necessary to implement a better morphology observation technique and standardize the method. Houbara sperm quality was previously studied with techniques used routinely in breeding facilities, such as sperm concentration, motility score and sperm viability/morphology with eosin-nigrosin staining. Even though these techniques are suitable to evaluate a large number of samples daily, they provide limited information on sperm function. Using more advanced and objective techniques to evaluate houbara semen would provide more robust and reliable data and more insight on sperm function.

With regard to aging, hormonal changes associated with male age have not been studied. As a decline in sperm production is observed in older males (Preston *et al.* 2011), we can expect testosterone levels to decrease with age in houbara males.

In addition to mating order, the outcome of sperm competition is also correlated with sperm number. By producing more sperm, males can secure siring success when sperm competition occurs. However, producing higher sperm numbers is energetically costly and can lead to poorer investment in quality. Such a trade-off between sperm quantity and quality was never explored before in houbara. We can expect males producing a high number of sperm to have lower sperm quality. Moreover, we could also expect an increase in the strength of this trade-off for old males.

It is known that male age impairs fitness, but the impact of sperm senescence inside the male reproductive tract on fitness has not been explored. Female fertility period after one insemination corresponds to the time of sperm storage inside the female tract. As fertility decline after insemination, this suggests sperm aging inside female tract. We can also expect sperm aging during the storage inside male ducts. If sperm cells do suffer from ageing, we expect a sperm quality decline with extended storage time, and this should impact reproductive success. We also expect a negative interaction with male age, with old males suffering from a higher rate of sperm senescence. Moreover, the possible interactions between LMP, age, and sperm quality have never been explored. If age is a greater predictor of siring success than mating order, we could expect LMP to occur less when females mate last with old males. We could also expect higher fertilisation success for the first male in the insemination sequence if its sperm quality was better than the last male.

5. THESIS OBJECTIVES

In this context and to investigate reproductive senescence, post-copulatory selection, and their interaction, the first objective of this thesis was to acquire deeper knowledge of the reproductive biology of the North African Houbara Bustard. To this purpose, the anatomy and histology of both male and female reproductive tracts during breeding and non-breeding seasons were studied and described to explore mechanisms underlying sperm competition. A standardized sperm morphology description and classification of defects will be established. Overall studying anatomy, histology and sperm morphology will give a better understanding of houbara reproductive biology.

The second objective was to explore mechanisms of post-copulatory selection. We aimed to test the different LMP hypotheses by using two fluorescent staining to visualize and differentiate sperm from different inseminations inside the SSTs (previously located with the anatomy study). Depending on the mechanism(s) acting in houbara, we can expect sperm displacement, stratification, segregation and/or passive sperm loss over time.

The third objective was to investigate male reproductive senescence and male age's effect on LMP and was divided into four parts:

(1) To investigate male age's effect on testosterone production across the breeding season, serum testosterone levels were measured throughout the season in old and young male breeders. Concentrations measured were confronted to display frequency, ejaculate collection success and sperm number to explore possible interactions of age, hormonal status, reproductive behaviour, and gametes production.

(2) Aiming to explore male age impact on sperm traits and possible trade-offs between sperm number and the different sperm traits, we evaluated different aspects of sperm function using objective techniques. Thus, sperm viability, mitochondrial potential, and chromatin structure were assessed with flow cytometry, multiple motility and velocity parameters were measured using a Computer Assisted Sperm Analysis (CASA) system, and sperm morphology was analysed using a classification adapted for bustard sperm.

(3) We aimed to test male age, sperm age, and their interaction on sperm quality and reproductive success. We investigated sperm ageing in the male tract by controlling the collection interval timing for young and old males. These ejaculates were evaluated for various sperm quality parameters: viability, ROS production, motility, velocity, and morphology. Females were inseminated, and hatching rate, chick's growth, and survival were recorded.



(4) We aimed to study LMP in controlled experimental conditions to unravel the importance of male age, mating order, and sperm quality in fertilization success. Females were inseminated in sequence with ejaculates, without control over quality, of old and young males. Laid eggs were collected, and paternity was assigned to the offspring.

To achieve these 3 main objectives, this thesis work is organised in 6 chapters:

For objective 1

Chapter 1: Sperm morphology, macro and microscopic description of male and female reproductive tract in the North African houbara bustard (*Chlamydotis undulata undulata*)

Anatomy and histology of male's and female's reproductive organs were described and compared during the breeding and non-breeding season. Morphometry and normal and abnormal sperm morphology were described using phase contrast and electron microscopy. A first classification for houbara sperm was proposed.

For objective 2

Chapter 2: Exploring the mechanisms underlying the last male precedence in the North African Houbara Bustard

We used competitive inseminations to explore sperm storage dynamics inside sperm storage structure (SSTs) of the oviduct and investigate the last male precedence mechanism. A combination of two fluorescent staining (Hoechst 33342 and MitoTracker™ Red FM) was used to visualize, differentiate and quantify sperm from two males inside SSTs. The impact of the fluorochromes on motility was tested prior to inseminations.

For objective 3

Chapter 3: Testosterone rise precedes the peaks of sexual display and higher sperm quality in adults and older captive North African houbara bustards (*Chlamidotys undulata undulata*)

We investigate serum testosterone levels across season in young and old age groups and their association with displaying frequency, semen collection success and sperm number ejaculated. Serum testosterone concentrations were measured across breeding season, from December to June by using ELISA dosage.

Chapter 4: Phenotypic trade-offs between sperm quantity and quality across age groups in a polyandrous bird, the North African Houbara Bustard

We explored a potential trade-off between sperm quantity and quality. We examined the pattern of covariation of multiple sperm quality traits (motility, velocity, progressivity, viability, mitochondrial potential, ejaculate volume, DNA fragmentation and condensation, round cells concentration and morphology) with number of sperm ejaculated. We explored this covariation patterns in young and old bird, to estimate the covariation independently of age, and to assess whether age increases the strength of the expected trade-off between sperm quantity and quality.

Chapter 5: Pre-but not post-meiotic senescence affects sperm quality and reproductive success in the North African Houbara Bustard

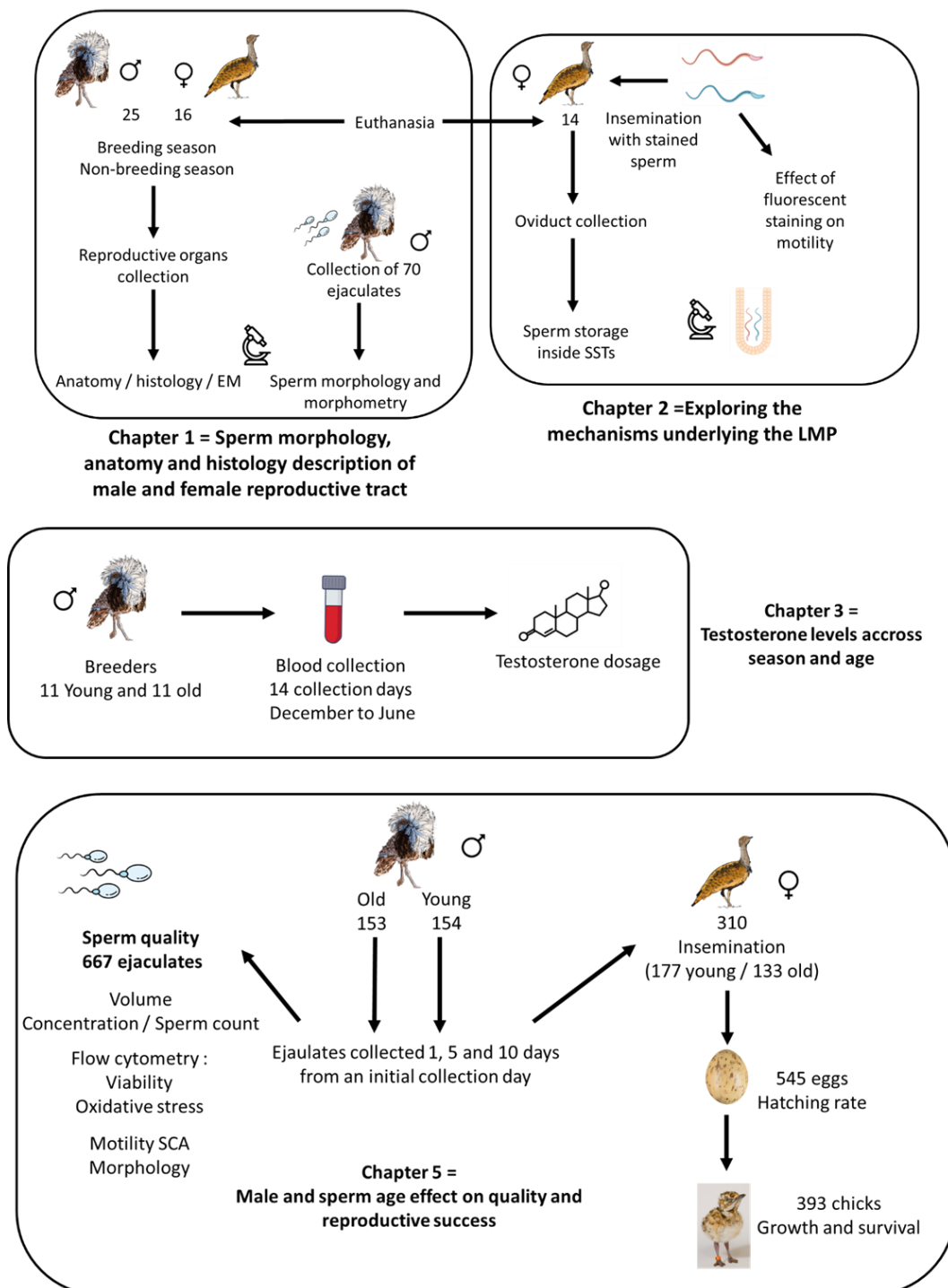
We tested male and sperm senescence effect on multiple sperm quality parameters (sperm viability, motility, ROS level and morphology) and on reproductive success. Young and old males were collected at different time interval (1, 5 and 10 days) apart from an initial collection day. Ejaculates were assessed for sperm quality and used for artificial insemination. Hatching success, chick growth and survival were recorded to assess reproductive success.

Chapter 6: Disentangling the importance of ejaculate attributes, age and mating order in a species with a last male precedence effect

To explore male age interaction with last male precedence, we performed competitive insemination using raw ejaculates in sequence. One group of females were inseminated first with sperm of young male and 48 hours after with an old (YO) and a second group were inseminated first with an old male and secondly with a young one (OY). Ejaculates used for insemination were assessed for different sperm quality parameters (sperm number, viability, motility, velocity and morphology). Mothers, potential fathers and offsprings were genotyped to assign paternity.

MATERIALS AND METHODS

The common methods from the sixth chapters will be presented in these sections, and specific methods and experimental design of each study will be described in detail in the “Materials and methods” session of each chapter. A diagram summarizing the different experimental designs and main methods used for each chapter is represented in Fig. 14.



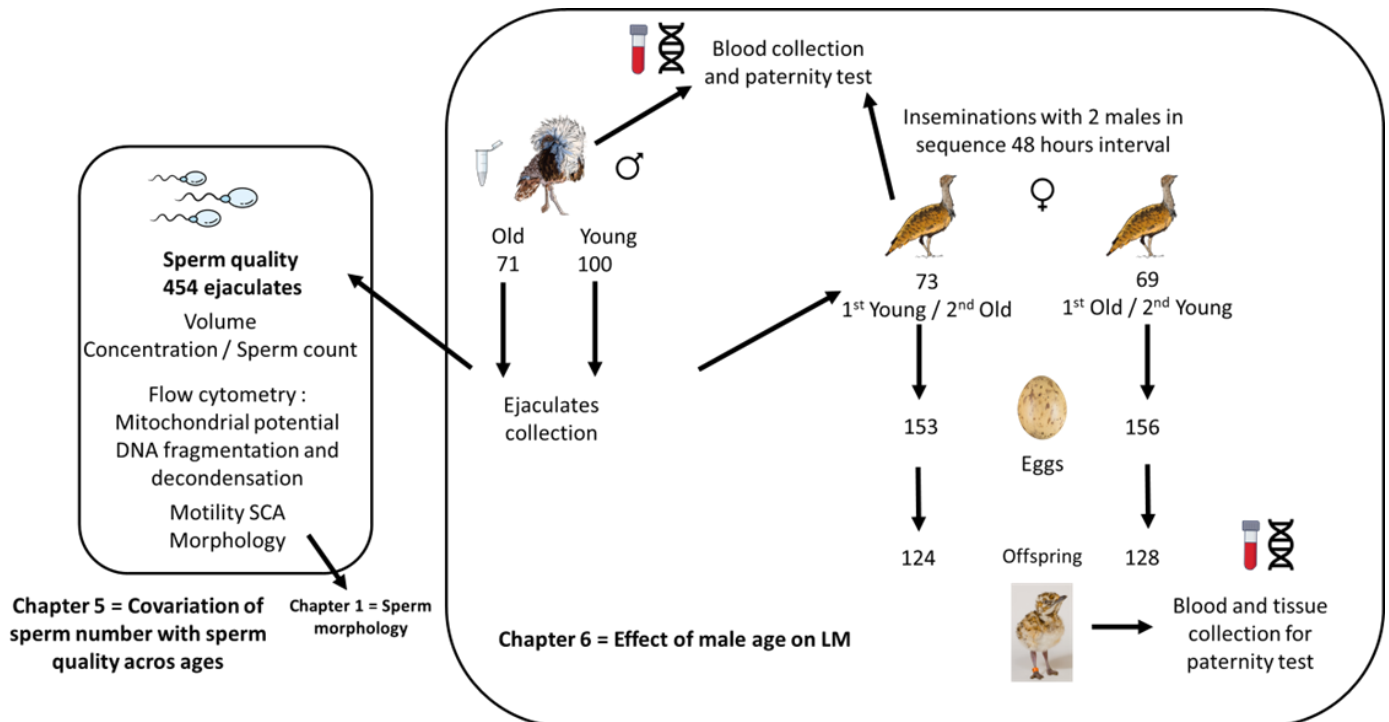


Figure 14: Schematic summary of the experimental designs and methods used for each chapter of the thesis.

1. HOUBARA CONSERVATION BREEDING

All birds used in this thesis work were part of a conservation breeding program at the Emirates Center for Wildlife Propagation (ECWP) in eastern Morocco, birds were allocated in two centers in the Fez-Boulemane department (GPS location (latitude, longitude): Missouri center: (33.007°/-4.098°), and Enjil center: (33.147° -4.578°), aiming at reinforcing natural populations of the North African Houbara Bustard (Lacroix 2003). All chicks are produced by artificial insemination (AI) under strict genetic management to ensure genetic diversity (Rabier et al. 2020; Loïc Lesobre 2008). The breeding flock counts a total of 7713 breeders, 2961 males, and 4752 females, currently producing about 20,000 chicks annually, used for conservation translocation and supplementation for hunting across the species range. Breeders are housed individually in 4 square meters cages whereas birds intended for release are kept in 30 by 9 meters aviaries. More details on the conservation program are available at www.houbarafund.org.



2. EXPERIMENTAL ANIMALS

All birds used for semen collection, blood collection and insemination were part of the breeding flock. All birds used for the anatomic, histological and sperm storage studies were born in captivity and were of generation F3 or more. They were deemed to be culled for the management of the captive flock; no bird that could have either been released in the wild or kept as a future breeder was euthanized. All birds received commercial specially formulated dry food pellets, mealworms, and crickets as food.

3. SEMEN COLLECTION

Males are collected in the morning by a trained technician using a dummy female. After displaying, the male mounts the dummy and starts copulating, at this moment a glass petri dish is used to collect the ejaculate by pressing it gently on the male cloaca during ejaculation (Fig. 15). The semen is immediately transferred to a 2 mL cryotube and maintained at room temperature. Ejaculates are directly assessed for motility (using a mass motility index ranging between 0 and 5, see Vuarin et al. 2019a for details), volume and sperm concentration (by spectrophotometry) and diluted (1:1) in Lake 7.1 (Lake and Ravie, 1984).



Figure 15: Breeding technician collecting a male using a dummy female.

4. ARTIFICIAL INSEMINATION (AI) PROCEDURE

To evaluate reproductive status, females are regularly checked for cloacal development. Insemination is performed when they are developed and ready to lay, usually 3 to 6 days before clutch



initiation. Females are held head down and cloaca is gently opened using a forceps. Sperm is then slowly delivered about 1 cm inside the vagina using an insemination tube mounted on a pipette (Fig. 16; Saint Jalme et al., 1994).

All females were inseminated in the morning with a minimum volume of ejaculate of 50 μ L and a maximum of 130 μ L. Depending on the experiment, females were inseminated before the first clutch of season or between the first and the second one with no previous insemination during the season and with or without thresholds for motility and sperm number (see chapters 5 and 6).



Figure 16: Artificial insemination (AI) procedure done by breeding technicians.

5. EGG COLLECTION, INCUBATION AND CHICKS REARING

Eggs were collected daily, disinfected and placed in artificial incubation for 21 to 24 days. Collected eggs showing defects like damaged shells or that were underweight were not incubated. Fertility assessment was performed at day 8 of incubation by candling method. An egg was considered fertile when a sign of embryo development was observed such as a developed air chamber, visible embryo, or blood vessels. If no signs of fertility were observed, the eggs were opened to visually discriminate between early embryonic death (ED) and “clear-ED”. The egg fertility was considered “clear-ED” when it was not possible to distinguish unfertile eggs from early embryonic death. Eggs that did not hatch after 24 days of incubation were also open and embryo development evaluated. When the embryo development stopped during the second and the last third of incubation, the egg is classified as mid-death (MD) and late-death (LD) respectively. Hatched viable chicks were reared and manually fed in cages until 35 days and after transferred to outdoor aviaries.



6. BLOOD AND TISSUE COLLECTION

Blood samples were collected from adult males for testosterone dosage and from adult male and female breeders for paternity analysis. Blood from chicks and tissue from embryos were collected for paternity analysis. Blood samples from adults were collected from the jugular vein or from the brachial vein with a 3 mL syringe and a blue needle. Blood samples from chicks were collected using 20 μ L heparin free capillaries (Hirschmann, Germany). Tissue samples (muscle, 1mm³) were collected during embryonic death evaluation.

7. SPERM QUALITY ANALYSIS

7.1 Flow cytometry analysis

Flow cytometry analysis was performed using a Guava easycyte 5HT (IMV, L'Aigle, France) flow cytometer equipped with a 150-mW blue argon laser (488 nm) and 3 colour photomultipliers green (525/30 nm), yellow (583/26 nm) and red (695/50nm). Data were analysed using the guavaSoft IMV software (IMV, L'Aigle, France). Before starting sperm analysis, sperm autofluorescence was tested. Indeed, every cell emits fluorescence signal to some extent. Extenders used to dilute semen can increase the autofluorescence signal in sperm cells and so interfere with flow cytometric analysis. Thus, it is important to test the effect of dilution media on sperm autofluorescence signal before conducting flow cytometry analysis (Carreira et al., 2012). Three extenders were tested for autofluorescence induction on sperm cells: Lake, PBS/BSA 3% and PBS/BSA 1.5%. Twenty microliters of sample (mix of sperm) were diluted in 180 μ L of each extender in a 96 wells plate and the plate was read for green, yellow and red fluorescence. Sperm showed the same level of fluorescence signal with the 3 extenders and autofluorescence is considered not induced when no signal above 10² units in the logarithmic x axis of the histograms were detected in all the three-color channels. Extender PBS/BSA 1.5% was chosen for the assays as it was previously used before on flow cytometry analyses on North African houbara sperm.

7.1.1 Viability

The viability or membrane integrity was assessed using Easy Kit™ 1 viability (IMV technologies), which contains propidium iodide (PI) and SYBR-14 dye dried and pre-loaded in 96 rounds bottoms well plates. PI exclusively enters damaged plasma membranes and binds to nucleic acids and when excited at 488nm emits red fluorescence at 617 nm (Gillan *et al.*, 2005). SYBR-14 dye is membrane permeable and emitting green fluorescence at 518 nm when bound to nucleic acids (Garner et al. 1994), when



sperm membrane is permeable by PI the red signal overtakes the fluorescent signal of the SYBR green. Twenty microliters of diluted (1:10) sample were transferred in the kit plate with 180 μL of PBS/BSA 1.5% and mixed. Negative control was made by incubating 20 μL of samples mix with 180 μL of Triton X 1% in 1.5mL cryotubes for 15 minutes in the dark at room temperature. Triton X is a detergent that permeabilizes the membranes. Samples were washed by centrifugation for 5 minutes at 1000g and resuspend in 180 μL of PBS/BSA 1.5%. Finally, 200 μL of the treated samples were loaded to the 96 wells kit plate and mixed. Samples were analysed after 15 minutes of incubation in the dark at RT on a plate shaker (Green, 525/30 nm, and red fluorescence, 695/50 nm, 10000 gated events).

7.1.2 Mitochondrial potential

Mitochondrial potential was assessed using EasyKit™ 2 Mitochondrial activity (IMV, L'Aigle, 131 France), which contains JC-1 dye dried and pre-loaded in 96 rounds bottoms well plates. JC-1 fluorochrome accumulates in monomer form in depolarized mitochondria emitting green fluorescence at 529 nm. In response to high mitochondrial membrane potential, monomers aggregate and emit orange fluorescence at 590 nm. First JC-1 dye was resuspended by adding 5 μL of DMSO and mixing. Then 20 μL of diluted (1:10) sample were diluted in 180 μL of PBS/BSA 1.5% and transferred to the kit plate. For positive control 0.25 μL of Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) (50 mM, from MitoProbe™ JC-1 Assay Kit (Molecular Probes, USA) was added to 200 μL of diluted (1:10) sample, incubated 5 minutes at 37°C and transferred in the kit plate. CCCP is mitochondrial membrane potential disrupter that inhibits oxidative phosphorylation and thus provokes depolarization of mitochondrial membranes. Samples were analysed after 30 minutes of incubation in the dark at RT on a plate shaker (green, 525/30 nm, and yellow, 583/26nm, 10000 gated events).

7.1.3 Oxidative stress

Intracellular sperm oxidative stress was measured in live cells using a combination of Dihydroethidium (DHE, 2mM in dimethyl sulfoxide (DMSO), Sigma-Aldrich, St Louis, USA), which emits between 570 and 600nm orange fluorescence when reacting with superoxide anions O_2^- , a reactive oxygen species (ROS) (Zhao et al. 2005), and SYBR-14 (Sigma-Aldrich) to measure percentage of living and oxidised sperm cells (out of 10,000 cells). In a 96 well plate 180 μL of PBS/1.5% BSA, 20 μL of diluted semen (1:10 in PBS/BSA), 1 μL of SYBR-14 (Sigma-Aldrich, St Louis, USA, 1 mM stock solution, 20 μM in DMSO work solution) and 2 μL of DHE (2mM in DMSO) were added. Negative control was performed incubating sperm with hydrogen peroxide for 15 minutes at 40 °C prior to analysis. Samples were analysed after 15 minutes of incubation in the dark, at room temperature (green, 525/30 nm and orange, 583/26 nm), 10000 gated events).



7.1.4 Sperm Chromatin Structure Assay – DNA fragmentation and chromatin decondensation

The DNA fragmentation and compaction was measured with the Sperm Chromatin Structure Assay (SCSA) with modifications (Evenson et Jost 2000). In this technique, acridine orange (AO), a metachromatic DNA stain, intercalates into double-strand DNA and emits green fluorescence (515–530 nm) and stacks on single-strand DNA and emits red (>630 nm) upon excitation from a 488 nm laser. The extent of single and double DNA strand breaks (DNA fragmentation index, %DFI) and level of excess nuclear histones (high DNA stainable sperm, %HDS) are simultaneously measured in individual sperm (Evenson 2022). The DNA fragmentation index (DFI%) is obtained with the ratio of red fluorescence over the total fluorescence (green and red, see Fig. 17 for an example of plots and results on the guavaSoft IMV software).

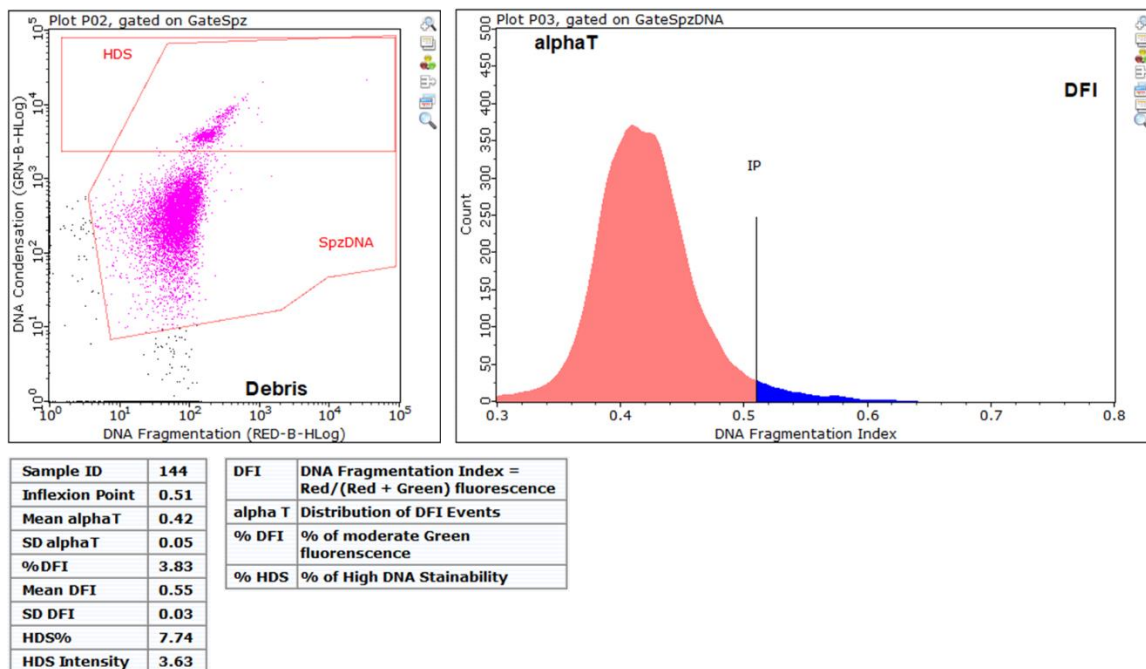


Figure 17: Example of results output for SCSA on guavaSoft IMV software.

A phosphate-citrate buffer was prepared by adding 370 ml of citric acid buffer (0.1M) and 8.77g of NaCl to 630 ml of Na₂PO₄ buffer (0.2M) and then stored at 4°C. An AO staining solution (3 µg/ml) was made with AO stock solution (Sigma, 1mg/ml) and the phosphate-citrate buffer. In a 96 well plate, 180 µl of AO staining solution was added in each well with 20 µL of diluted semen (1:10 in PBS/BSA 1.5%). For HDS% positive control 20 µL of control sample were incubated with 180 µL of DTT 2nM/ Triton 0.5% for 10 minutes in the dark on a plate shaker. After incubation, the sample was centrifuged for 5 minutes at 1000g, resuspended in 180µL of AO solution and 200 µL were transferred in the plate.



Samples were analysed after 3 minutes of incubation in the dark at RT on a plate shaker (green, 525/30 nm and red, 695/50 nm, 10 000 gated events).

7.2 Sperm motility

Sperm motility was assessed with the Sperm Class Analyzer software (SCA, Microptics, Barcelona, Spain). Four microliters of diluted sample (1:10) were placed on a 20 μm depth disposable chamber (Proiser, Arquimea, Collado Villalba, Spain) under a negative phase contrast microscope (Nikon E200-LED, Tokyo, Japan, to objective x10, -ph). A maximum of 30 fields were analysed as to reach 500 sperm (see table I for detailed SCA settings). Multiple motility and kinetics were recorded (table II and Fig. 18) and percentage of motile sperm, sperm progressivity and velocity [curvilinear velocity, VCL ($\mu\text{m}/\text{s}$)] were analysed.

Table I: SCA settings

Microscope		Catpure	
Calibration name	10x	Analysis timeout	15
Calibration value ($\mu\text{m}/\text{pixel}$)	0,420918	Box size (pixels)	152
Capture method	Ph-	Check image	Yes
Grid distance (μm)	100	Frame rate (fps)	50
Disposable		Number of images	25
Disposable	Proiser	Preview	one second
Depth (μm)	20	Resolution	Low
SS correction factor	1.3	Safe Mode	Yes
		Style	Automatic
Parameters		Finish	
Area (μm^2)(min)	5	Sperm to analyse	500
Area (μm^2)(max)	190	Fields to analyse	30
Drifting ($\mu\text{m}/\text{s}$)	0	Minimum spem to analyse	200
Static ($\mu\text{m}/\text{s}$) <	10	Minimum fields to analyse	3
Slow-Medium ($\mu\text{m}/\text{s}$)	50	Warning (%)	20
Rapid ($\mu\text{m}/\text{s}$) >	100		
Progressive (STR>)	70		
Connectivity (pixels)	18		
VAP points (pixels)	5		
VCL/VAP	VCL		



Table II : Description of the main motility and kinematics parameters analysed by SCA software (specifications available on the manufacturer's website: <https://www.micropticsl.com/products/sperm-class-analyzer-casa-system/main-modules/sca-motility-concentration/>)

Parameters	Description
Total motile %	Percentage of moving sperm in all captured sperm cells
Progressive %	Percentage of sperm moving forward in a straight path = Percentage of sperm exceeding STR defined thresholds
VCL, curvilinear velocity (m/s).	Time-averaged velocity of a sperm head along its actual curvilinear path, as perceived in two dimensions in the microscope. A measure of cell vigour.
VSL, straight-line (rectilinear) velocity ($\mu\text{m/s}$).	Time-averaged velocity of a sperm head along the straight line between its first detected position and its last.
VAP, average path velocity ($\mu\text{m/s}$).	Time-averaged velocity of a sperm head along its average path. This path is computed by smoothing the curvilinear trajectory according to algorithms in the SCA Motility module.
ALH, amplitude of lateral head displacement (μm).	Magnitude of lateral displacement of a sperm head about its average path. It can be expressed as a maximum or an average of such displacements.
LIN, linearity.	The linearity of a curvilinear path, VSL/VCL .
WOB, wobble.	A measure of oscillation of the actual path about the average path, VAP/VCL .
STR, straightness.	Linearity of the average path, VSL/VAP .
BCF, beat-cross frequency (Hz).	The average rate at which the curvilinear path crosses the average path.

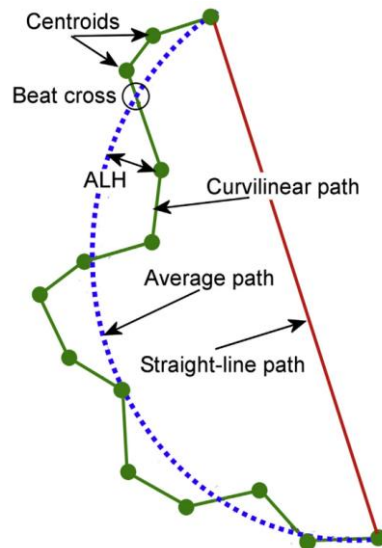


Figure 18: Schematic representation of kinematics parameters analysed with CASA system (From Amann and Waberski, 2014): A centroid point is provided for each sperm cell by the processing of the first frame captured and the most probable centroid in successive frames is deduced of each cell location. The curvilinear path is provided by connecting the centroids of one sperm together.

7.3 Sperm morphology

Semen samples were fixed by adding 5 μL of 4% buffered paraformaldehyde to 10 μL of semen diluted in PBS/BSA 1.5% (1:10). Then, 6 μL of the suspension were placed on a slide with a coverslip (wet mount) and observed under a phase contrast microscope (Nikon E200-LED, Japan; objective x100, Phase contrast, oil immersion). For each sample, 100 sperm cells were classified according to 8 categories adapted from both Bloom and Alkan's previous work (Alkan *et al.* 2002; Bloom 1973b): acrosome loss, abnormal head, swollen head, midpiece defects, tail defects, cytoplasmic droplet, teratogenic sperm (double head or tail) and normal. Sperm morphology classification is described in detail in Chapter 1.

8. REPRODUCTIVE ORGAN COLLECTION AND HISTOLOGY PREPARATION

To collect reproductive organs, euthanasia was performed by an ECWP veterinarian (1 mL of T61 intravenous, MSD, 200 mg Embutramide, 50 mg Mebezonium Iodide, and 5 mg Tetracaine Hydrochloride) (Baker-Cook *et al.* 2021). A total of 55 sexually mature houbara adults ($N_{\text{males}} = 25$, age mean_{males} \pm SD = 5.16 ± 0.37 , Min-Max = 5-6; $N_{\text{females}} = 30$, age mean_{females} \pm SD = 5.6 ± 0.72 , Min-Max = 5-7) of both sexes were euthanized. The coelom was opened, and the reproductive organs removed.



Organs were photographed, measured, fixed according to the different analyses (Histology or Electron microscopy, Chapter 1) or transferred in PBS 1X (oviduct for Chapter 2).

9. ETHICS STATEMENT

The breeding program runs under the approval of the Moroccan Ministry of Agriculture and a mandate independent veterinarian (mandate number: 534-98), providing control of the well-being of the birds. Onsite veterinary facilities ensure the best possible care for sick or injured birds by a team of expert veterinarians, and standards from sanitary authorities are regularly controlled. Experiments are conducted ethically, and each breeding protocol is developed while ensuring appropriate and ethical treatment of birds. To minimize stress during capture, birds are fitted with hoods during manipulation. Facilities are designed to ensure the well-being of the birds and prevent injuries.



RESULTS

CHAPTER 1: SPERM MORPHOLOGY, MACRO AND MICROSCOPIC DESCRIPTION OF MALE AND FEMALE REPRODUCTIVE TRACT IN THE NORTH AFRICAN HOUBARA BUSTARD (*CHLAMYDOTIS UNDULATA UNDULATA*)

Léna Meunier ^{a b}, Gabriele Sorci ^b, Caroline Silva Vieira ^{a c}, Yves Hingrat ^a, Michel Saint Jalme ^a, Janaina Torres Carreira ^a

^aReneco International Wildlife Consultants LLC, Abu Dhabi, United Arab Emirates

^bBiogéosciences, UMR 6282 CNRS, Université de Bourgogne Franche-Comté, Dijon, France

^cFederal University of Minas Gerais, UFMG, Belo Horizonte, MG, Brazil

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Highlights

- First anatomic and histologic description of a bustard reproductive tract
- Regression of male and female's gonadal activity during the non-breeding season
- Presence of melanin pigments in the testis, ovary, and oviduct.
- The first description of houbara sperm ultrastructure and morphology classification

Abstract

Ex-situ conservation programs are useful tools for preserving endangered species, and knowledge of their reproductive physiology is essential for achieving conservation objectives in captive breeding programs. The North African Houbara Bustard (*Chlamydotis undulata undulata*) is a vulnerable bird species bred in captivity to restore its natural populations. To better understand the reproductive biology of this species, we first described the anatomy and histology of the reproductive tracts of male and female North African houbara bustards. We described and classified the normal and abnormal sperm morphologies for each species. The reproductive tracts of males and females were collected



during the breeding season (BS) (21 males and 14 females) and the non-breeding season (NBS) (four males and two females). Pictures and measurements of the testes and oviducts were taken, and testis colour was graded from 1 (black) to 6 (white). Organs were fixed and processed for histological analysis, scanning electron microscopy (SEM), and transmission electron microscopy (TEM). The testis, epididymis, oviduct size, testicular capsule thickness, seminiferous tubule (ST) diameter, and histological layers of the oviduct were compared between the BS and NBS groups. Sperm morphometry was evaluated using fixed ejaculates (n=22, from 22 males). Ejaculates (n=417, 166 males) were collected and fixed during the BS, and their morphology and morphometry were evaluated using phase-contrast microscopy. Ejaculate mixes (n=12) were fixed and analysed using SEM and TEM. Overall, the anatomical structure and histology of the male and female reproductive tracts were similar to those of other avian species. The testes were symmetric, and a regression of testis and oviduct size, the thickness of the mucosa and serosa of the oviduct, and ST diameter ($p < 0.001$) were observed during NBS compared with those during BS. Sperm storage structures are located inside the oviduct at the uterovaginal junction and infundibulum. Melanin granules were observed in the interstitial space of the seminiferous epithelium, which explains the testis pigmentation. Melanin was also found in the ovaries and the uterus. Houbara sperm have an elongated nuclear form and a round-shaped acrosome lacking perforatorium and rostrum. Most analysed samples were limitrophe normospermic (normal = 68.5 ± 14.7 %), houbara sperm morphology could be classified into eight categories: normal sperm, acrosome, head, midpiece, tail, and teratogenic defects, swollen head, and cytoplasmic droplet. This study provides the first anatomical and histological descriptions of the male and female reproductive tracts of the North African houbara bustard. Further investigation into the functional role of melanin in the testes is warranted.

Keywords: houbara bustard, seasonal breeding, anatomy, histology, reproductive organs, melanin, sperm ultrastructure, sperm morphology



Introduction

There are over 11,000 bird species, almost twice as many as mammals; however, the study of avian reproductive biology is still less advanced than that of mammalian species. Birds use a wide variety of reproductive strategies and mating systems, including monogamy, polygamy, seasonal breeding, gonadal anatomy, and gamete traits. The reproductive biology of non-passerine birds has been documented for domestic species, such as chickens (*Gallus gallus domesticus*), turkeys (*Meleagris gallopavo*), quails, and ducks, (Hodges 1974; King & McLelland 1984; Pollock & Orosz 2002; Jamieson 2007b; Johnson 2015; Vizcarra *et al.* 2015) and other non-domestic species of economic interest, such as ratite birds, the Greater Rhea (*Rhea americana*), Ostrich (*Struthio camelus*), and Emu (*Dromaius novaehollandiae*) (Budras & Meier 1981; Soley JT 1992; Sales 2007; Parizzi *et al.* 2008; Freneau *et al.* 2016). However, knowledge of wild bird species is scarce, and only a few birds, such as raptors, cranes, and penguins, have been studied for conservation breeding purposes (Archibald 1974; Ellis *et al.* 1996; Blanco *et al.* 2000; Blanco *et al.* 2002; O'Brien & Robeck 2014; Rodler *et al.* 2015; O'Brien *et al.* 2016a; O'Brien *et al.* 2016b; Mafunda *et al.* 2021).

Ex-situ conservation breeding programs are useful tools for preserving and restoring endangered species (Blanco *et al.* 2009). As these programs rely on the breeding of captive animals, comprehensive knowledge of the reproductive biology and physiology of the species is paramount. For instance, seasonal breeders, such as the Emu and Ostrich, have different breeding periods depending on their geographic location, (Malecki *et al.* 1998; Gee *et al.* 2004) with consequences for the management of captive breeding. Knowledge of seasonal variations in reproductive organs and hormones can be useful for improving captive breeding strategies, as shown in African penguins (*Spheniscus demersus*) (Mafunda *et al.* 2021). Having reference to healthy reproductive organs is also helpful in assessing the reproductive status of individuals and managing reproductive pathologies in breeders (Clubb *et al.* 2015; Bailey & Lierz 2017). Baseline references for semen parameters such as volume, concentration, viability, and motility are essential for successful artificial insemination (AI) programs (Blanco *et al.* 2009). Sperm morphology is an important parameter that contributes to the definition of sperm quality. The percentage of sperms with normal morphology correlate with fertilisation success in roosters (Łukaszewicz 1988; Łukaszewicz *et al.* 2008), stallions (Love 2011), dairy bulls, (Al-Makhzoomi *et al.* 2008), and humans (Enginsu *et al.* 1991). The sperm morphology can also indicate dysfunctional spermatogenesis (Ceyhan *et al.* 2005). The general sperm ultrastructure has been documented in other avian species (Jamieson 2007a). However, a description and homogenous classification of the morphological defects in avian sperm are lacking. The morphological classification of avian sperm has not been standardised for livestock, and literature is scarce; (Alkan *et al.* 2002; Bloom 1973a; Łukaszewicz *et al.* 2008; Du Plessis & Soley 2011, 2014; Feyisa *et al.* 2018), studies often use



the classical classification system that was initially described for bovine semen (Bloom 1973a) and later adapted for avian sperm evaluation (Alkan *et al.* 2002).

The North African Houbara Bustard (*Chlamydotis undulata undulata*) is a medium-sized bird that inhabits the semiarid lands of North Africa from North Mauritania to Egypt. It belongs to the *Otidiformes* order, with 26 other species. Over the past decades, wild populations of the North African Houbara Bustard (*hereafter houbara*) have decreased due to habitat destruction and overhunting, and the species is now classified as “vulnerable” by the International Union for Conservation of Nature (IUCN 2016). Therefore, in the late 90s, a captive breeding program was initiated in Morocco to restore natural populations through conservation translocation.

The houbara is a polyandric seasonal breeder and has an exploded-lek mating system (Hingrat & Saint-Jalme 2005b), that is, the breeding season in the wild was observed from January to June. During this period males would perform courtship display in a large range area (exploded-lek) and females would choose and copulate with males on these sites, it has been reported that females possibly copulate with several males, since 60 % of the nests are composed of chicks sired by more than one father (polyandry) (Hingrat *et al.* 2004; Hingrat *et al.* 2007).

An interesting consequence of polyandry is the possible occurrence of post-copulatory sexual selection. Once the female mates with multiple males, sperm from different males can be stored inside specialised structures located on the female UVJ, called sperm storage tubules (SSTs) and compete for fertilisation. Houbara Saint Jalme *et al.* (Saint Jalme *et al.* 1994) reported fertile eggs 22 days after insemination. Post-copulatory sperm competition has been studied in captive houbaras, demonstrating the advantage of younger males in competitive mixed semen insemination (young versus old)(Vuarin *et al.* 2019a). Sperm can be stored for different periods in birds (Birkhead & Møller 1992), for example, from 6 days in the Ring Dove (*Streptopelia risoria*) (Zenone *et al.* 1979) and up to 72 days in turkeys (*Meleagris gallopavo*)(Lorenz 1950). The anatomy and histology of the houbara female tract have not been described previously. Thus, its capacity to store sperm and reproductive organ structures is presumptive and based on data from other avian species.

Nevertheless, in captivity, reproduction relies exclusively on AI and pairing is based on pedigree information to preserve genetic diversity and evolutionary resilience within the captive flock (Rabier *et al.* 2020). During the breeding season, ejaculates are collected by presenting a dummy female to a male, to which males will perform a courtship display, approach, mount the dummy, and ejaculate. The semen is collected by the technician that controls the dummy by pressing the male cloaca with a petri dish at the moment the bird ejaculates, and the semen collected is evaluated and later used for artificial insemination (Saint Jalme *et al.* 1994).



Houbara conservation breeding conditions, in which numerous birds are maintained under controlled conditions, provide valuable data for studying the reproductive biology of this species. Routine analysis of semen samples was conducted, focusing on fundamental parameters, such as volume, concentration, and mass motility index before their use in insemination. Semen quality data have been instrumental in various studies on sperm competition, reproductive success, and senescence (Preston *et al.* 2011; Preston *et al.* 2015; Vuarin *et al.* 2019a; Vuarin *et al.* 2019b; Vuarin *et al.* 2021; Sorci *et al.* 2023).

Recently, sperm parameters were studied with more details concerning senescence, and parameters, such as viability, oxidative stress, computer-assisted sperm analysis of motility, and sperm morphology were evaluated for the first time in the houbara (Meunier *et al.* 2022). However, to date, no data have been published on the reproductive anatomy and sperm ultrastructure in the *Otidiformes* order. Therefore, houbara is a model species that may serve as a reference for other endangered bustard species.

The aims of this study are to describe the anatomy and histology of the captive houbara reproductive tract during the breeding (BS) and non-breeding (NBS) seasons and normal spermatozoa morphology, and to propose the first classification of sperm morphological defects using both phase contrast and electron microscopy (EM) techniques.

Materials and methods

Houbara conservation breeding

All birds used in this study were part of a conservation breeding program at the Emirates Center for Wildlife Propagation (ECWP) in eastern Morocco, with the aim of reinforcing natural populations of the North African Houbara Bustard (Lacroix 2003c). All chicks were produced by AI under strict genetic management to ensure genetic diversity (Loïc Lesobre 2008; Rabier *et al.* 2020). The breeding flock includes 7713 breeders, 2961 males, and 4752 females, currently producing approximately 20,000 chicks annually, which are used for conservation translocation and supplementation for hunting across the species range. Breeders were housed individually in 4-m² cages whereas birds intended for release were kept in 30m × 9-m aviaries. Further details on the conservation program are available at www.houbarafund.org.

Experimental animals

All birds used for the anatomical and histological studies were born in captivity and of generation F3 or higher. They were deemed to be culled for the management of the captive flock; no bird that could have either been released into the wild or kept as a future breeder was euthanised. A



total of 41 sexually mature houbara adults (6 years old) of both sexes were euthanised. Males were kept in aviaries with eight males and eight females in individual cages.

A total of 417 semen samples from 166 male fully adult breeders (mean age= 10.45 ± 7.76 years old; males can produce offspring from 1 year old (Vuarin *et al.* 2021), were collected and used for the evaluation of sperm morphometry and morphology study. All the birds received specially formulated commercial dry food pellets, mealworms, and crickets.

Semen collection, sperm morphology classification, and morphometry

Semen was collected from dummy females for sperm morphology and morphometry. Briefly, after the display, the male mounted the dummy and started copulating, and a glass Petri dish was used to collect the ejaculate by pressing it gently on the male cloaca during ejaculation. The semen was immediately transferred to a 2 mL cryotube and maintained at room temperature (24°C) until processed for morphology and electron microscopy.

Semen samples were fixed by adding 5 μ L of 4 % buffered paraformaldehyde to 10 μ L of semen diluted in PBS/BSA 1.5 % (1:10). Then, 6 μ L of the suspension were placed on a slide with a coverslip (wet mount) and observed under a phase contrast microscope (Nikon E200-LED, Shonan, Japan; objective x100, Phase contrast, oil immersion). For each sample, 100 sperm cells were classified into eight categories adapted from Bloom and Alkan's previous study (Alkan *et al.* 2002; M; Bloom 1973b): acrosome loss, abnormal head, swollen head, midpiece defects, tail defects, cytoplasmic droplets, teratogenic sperm (double head or tail), and normal. The defects, when possible, were also compared with similar alterations using electron microscopy to better understand the structures involved.

For sperm morphometry, the ejaculates of 22 birds (mean age= 10.45 ± 7.76 years old) were fixed and observed under the phase contrast microscope. Images were taken (Nikon E200-LED, objective x40, phase contrast), and each part (acrosome, head, midpiece, and tail) of 20 spermatozoa was measured using Fiji software (Schindelin *et al.* 2012).

Reproductive organ collection and histology preparation

To collect the reproductive organs, euthanasia was performed by an ECWP veterinarian (1 mL of T61 intravenous MSD, 200 mg embutramide, 50 mg mebenzonium iodide, and 5 mg tetracaine hydrochloride (Baker-Cook *et al.* 2021). Thirty-five birds were euthanised and sampled during the BS (21 males and 14 females) and six during the NBS (four males and two females). Testes and epididymal structures were described and measured using a pachymeter, whereas oviduct segment photographs were used to measure the organs using Image J Fiji software version 1.53 (Schindelin *et al.* 2012). The colour of the testes was visually assessed using a colour grading scale (1–6, from black to white; Fig.1).



Histological samples were fixed in Bouin's solution (three parts acetic acid to five parts formalin and 15 parts saturated picric acid 2g/L in water) for 12 H and then transferred to 70 % alcohol (replaced three times every 24 h). For each male (BS, n=20; NBS, n=4), the right or left testis, epididymis, and different ducts were fixed. For females (BS, n=6; NBS, n=2), a fragment (1–2 cm) of seven different segments (the vagina, UVJ, uterus, isthmus, magnum, infundibulum, and ovary) was fixed.

Female and male reproductive tract samples were processed for histological analysis following standard procedures(2019). Samples (5-mm thick) were embedded in paraffin and 3- μ m cuts were stained with haematoxylin and eosin(Luna 1968). Slides were observed under a microscope (Olympus BX41, Tokyo, Japan) and photographed using (Olympus DP21 camera and CellSens software version 1.12, Japan).

To observe fresh tissue fragments of UVJ, cuts were transferred into phosphate-buffered saline (PBS). Mucosal folds containing SST were dissected and observed under a stereomicroscope (MZ9.5; Leica, Heerbrugg, Switzerland) within an hour of euthanasia. Images were captured using Leica Application Suite software EZ software (version 3.2.1, Leica, Switzerland).

Histologic measurements

The seminiferous tubule (ST) diameter and albuginea thickness were measured for each male using both testes (during the NBS) and either the left or right testis (during the BS). The diameter of ten round tubules per testis and the thickness of ten sections of the albuginea capsule were measured in males, and the thickness of ten sections of each layer of each oviduct was measured in females. Measurements were made on photomicrographs using the Fiji software (Schindelin *et al.* 2012).

EM preparation

Samples collected for EM were fixed in a solution composed of Milloning's phosphate buffer (0.13M pH 7.4) and 2,5 % glutaraldehyde (Electronic Microscopy Sciences, USA). Fragments (1 mm³) of either the right or left testes of males and the infundibulum and UVJ of females were collected.

For EM sperm sample preparation, 12 sperm mixes were prepared by combining in each of the 12 tube ejaculates from four different males (48 ejaculates total, mean age = 8.79 \pm 5.72 years, minimum sperm number 10 million cells each mix were centrifuged (5 min, 1000 \times g) to obtain visible pellets. The pellet was washed twice in Milloning's phosphate buffer (0.13M pH7.4) and resuspended in EM fixative solution. EM samples were kept for 1 h at room temperature and then stocked at 4°C until analysis.



Transmission Electron Microscopy (TEM)

Tissue samples were washed twice with PBS before fixation with osmium tetroxide 4 % (Electronic Microscopy Sciences). Samples were then dehydrated in an increasing ethanol concentrations (35 to 100 %) and embedded in Spurr's resin. Sperm samples were transferred into plastic capsule moulds, and tissues were transferred into silicone block moulds. After polymerisation, semi-thin sections (500 nm) were cut using a PowerTome XL ultramicrotome (RMC Boeckeler, USA) equipped with a diamond knife (Diatome 35°, Switzerland) and stained with toluidine blue to check for the presence of tissue/sperm. Ultrathin sections (60 nm) were cut and placed on formvar-coated copper grids. All grids were contrasted with 2.5 % uranyl acetate, and grids of tissues were contrasted with Reynolds lead citrate. Finally, the grids were examined under a transmission electron microscope (Hitachi H7700, Japan) operating at 60 kV.

Scanning Electron Microscopy (SEM)

Fixed tissues were washed three times in PBS (pH 7.2, 0.1 M) and dehydrated in an increasing ethanol concentration (50 to 100 %). Tissues were transferred inside microporous capsules (microporous specimen capsules 30 µm, EMS) and critical point dried in liquid CO₂ (12°C, Leica EM CPD 300, Germany). Sperm mixes were washed twice in PBS (pH 7.2, 0.1 M), resuspended in distilled water, and transferred on a membrane (Isopore membrane filters 0.2 µm GTTP, Millipore, Germany) using a syringe and a filter holder (Swinnex, Millipore, Germany). Membranes were placed into a perforated 2 mL microtube, dehydrated in an increasing ethanol concentrations (50 to 100 %), and then critical point dried in liquid CO₂ (12°C, Leica EM CPD 300). The membranes and capsules were transferred to a scanning microscope holder covered with carbon-adhesive tape and coated with platinum (5 nm) using a Leica EM ACE600 Sputter Coater (Leica). Samples were observed at 15 kV using a scanning electron microscope (Hitachi SU3500), and pictures were taken.

Statistical analysis

Anatomical measurements of the testes and epididymis and histological measurements of ST were performed using the Student's t-test or Mann-Whitney U test. Histological measurements of the different oviduct layers were performed using the Mann-Whitney U test. All statistical analyses were performed using the Jamovi software (version 2.4, The Jamovi project 2023). The normality of distribution and homogeneity of variances were checked using the Shapiro-Wilk and Levene's test, respectively. Anatomical measurements of the oviduct were not statistically analysed. First, owing to the reduced length of the oviduct during the non-BS, it was impossible to distinguish between each segment; thus, only the full length of the oviduct was measured. Second, the sample sizes were unbalanced between the BS and NBS groups (BS, n=14; NBS, n=2). This unbalanced sample size was



due to experimental management reasons. Samples collected during the BS were also used for other parallel experiments (not presented here), explaining the higher number of euthanised animals. Only samples collected during NBS were used in the present study; therefore, the number of euthanised animals was limited.

Ethics statement

The breeding program was approved by the Moroccan Ministry of Agriculture and a mandate-independent veterinarian (mandate number: 534-98), providing control over the well-being of the birds. On-site veterinary facilities ensure the best possible care for sick or injured birds by a team of expert veterinarians, and standards from sanitary authorities are regularly controlled. Experiments were conducted ethically, and each breeding protocol was developed to ensure the appropriate and ethical treatment of birds. To minimise stress during capture, birds were fitted with hoods during manipulation. Facilities are designed to ensure the well-being of birds and prevent injuries.

Results

Female reproductive tract

Macroscopic description: Anatomy

The female reproductive tract of the North African Houbara Bustard was composed of a single ovary (left) and an oviduct, which includes five segments: the infundibulum, magnum, isthmus, uterus, and vagina (Fig. 2). For the females analysed, follicles of variable sizes, pre-hierarchical, pre-ovulatory, and post-ovulatory follicles were observed during the reproductive season, whereas the ovaries contained only small pre-hierarchical follicles (Fig. 3), which were not measured because there was no guarantee that all ovarian tissue was intact when removed from the bird. The total mean length of the oviduct during the BS was 420.48 ± 70.52 mm, during the NBS. The oviduct was reduced, with a length between 65 and 88 mm (mean 76.31 ± 16.47 mm), being unclear to distinguish the segments macroscopically. The measurements of the different oviduct segments are reported in Table I. No statistical analyses were performed because of the sample size. Previously described sites for sperm storage in other species (King *et al.* 1999; Blesbois & Brillard 2007; Waqas *et al.* 2016; Mohamed Nabil *et al.* 2022), such as the distal section of the infundibulum (neck) and the UVJ, were identified based on differences in tissue appearance and anatomical location (Fig. 4A and 4 B), and the dissected region characteristic folds were confirmed under a stereo microscope (Fig. 4C and 4D).



Microscopic description: Histology and EM

Ovary

The ovary was composed of an outer cortex, where follicles of different maturation stages were found, and an inner medulla made of smooth muscle, connective tissue, nervous system structures, blood vessels, and arteries supplying the cortex (Fig. 5A and 5B). During the BS, various stages of follicle maturation were observed, from primordial to post-ovulatory and atretic follicles (Fig. 5A, 5C, and 5D). The follicles were surrounded by a perivitelline membrane, and a single layer of granulosa cells, small white follicles, and more mature follicles presented with thecal layers: the theca interna and theca externa (Fig. 5D). Atretic follicles were identified by the presence of many vacuolated cells with lipid inclusions, and macrophages containing brown hemosiderin were observed (Fig. 5C). During the NBS, primordial, primary, and small white follicles were observed in the cortex (Fig. 5B). The cortex was covered with a single layer of cuboidal germinal epithelium (Fig. 5D). Melanin granules were observed inside the cortex, surrounding follicles, and inside the medulla in all females sampled during the BS and NBSs (Fig. 5A and 5B), and were sometimes visible between granulosa cells and the theca interna (Fig. 5D).

Infundibulum

The infundibulum composed two distinct regions: the funnel region closer to the ovary and the neck region closer to the magnum. The funnel mucosa was composed of long and short primary folds lined with pseudostratified columnar ciliated and non-ciliated cells. The submucosa was a fine layer of connective tissue highly irrigated by blood vessels. The tunica muscularis was very thin, composed of longitudinal smooth muscle fibres, and irrigated with blood vessels. The tunica muscularis was surrounded by the tunica serosa (Fig. 6A).

The neck mucosa is composed of ramified primary, secondary, and sometimes tertiary folds. These folds were thicker than those in the funnel region and were lined with simple tall columnar to pseudostratified columnar ciliated and non-ciliated cells. The lamina propria was much thicker than the funnel region and contained tubular structures lined with a simple unciliated columnar epithelium with basally oriented nuclei (Fig. 4E and 4G). These structures resemble SST of the UVJ; however, there was no evidence of sperm storage in this area on the analysed fragments. The submucosa was made of loose connective tissue and irrigated with blood vessels and the lamina propria. The tunica muscularis was thicker than the funnel region, composed of inner circular and outer longitudinal smooth muscle fibres, and surrounded by the tunica serosa (Fig. 6B and 6C).

Magnum and Isthmus



The mucosa of the magnum and isthmus was thicker than that of the infundibulum and consisted of a large primary fold lined with a pseudostratified columnar ciliated cell layer. The lamina propria was abundant in tubular glands of variable diameters lined with simple unciliated columnar epithelium with basally oriented nuclei. Mucus was observed in the lumen of some samples, resulting in the secretory activity of the mucosal cells. The submucosa was composed of loose connective tissue and irrigated with blood vessels. The tunica muscularis was made of inner circular and outer longitudinal smooth muscle fibres, irrigated with blood vessels, and surrounded by the tunica serosa (Fig. 7 and 8).

Uterus

The uterine mucosa presented with primary and secondary folds and a pseudo-stratified columnar ciliated cell epithelium. The lamina propria contained tubular glands of variable diameters, lined with a simple unciliated columnar epithelium with basally oriented nuclei. The submucosa was thick, composed of loose connective tissue, and highly irrigated with blood vessels. The tunica muscularis was thicker, made of inner circular and outer longitudinal smooth muscle fibres, and surrounded by the tunica serosa (Fig. 9).

UVJ

With the same cellular structure, the mucosa of the UVJ comprised long and ramified folds (primary, secondary, and tertiary). The lamina propria contained SSTs lined with a simple unciliated columnar epithelium with basally oriented nuclei. The submucosa was composed of loose connective tissue and irrigated with blood vessels. The tunica muscularis was thick, made of inner circular and outer longitudinal smooth muscle fibres, and surrounded by the tunica serosa. Melanin granules were also observed in the lamina propria, submucosa, and tunica muscularis (Fig. 4F).

Vagina

The mucosa of the vagina was composed of highly ramified folds (primary and secondary) lined with a pseudo-stratified columnar epithelium composed of ciliated and non-ciliated cells. The lamina propria did not contain tubular glands. The submucosa was made of loose connective tissue, irrigated with blood vessels, and has the same muscular structure as the UVJ. Melanin granules were also observed in the lamina muscularis (Fig. 10).

During NBS, different sections of the oviduct were made of the same layers, mucosa, submucosa, lamina muscularis, and serosa, when compared with the BS. The thicknesses of the different layers of all sections of the oviduct were measured during the BS and NBSs and are reported in Table II. A significant difference was observed in the mucosa and serosa thicknesses ($P < 0.001$),



which were thicker during the season, but not in the muscularis ($P= 0.290$) and submucosa thicknesses ($P= 0.998$) when BS and NBS measurements were compared.

Male reproductive tract

Macroscopic description: Anatomy

The male reproductive tract consists of two symmetric bean-shaped melanistic intra-coelomic testes located ventral to the kidneys, followed by two epididymides and two different ducts connected to the cloaca (Fig. 11A and 11C). No significant difference in testis dimensions was observed between the right and left testes, but they were longer and wider during BS in comparison with the NBS-harvested testes (Fig. 11B and 11D, Table III). As the epididymis was reduced in the NBS samples; it was not possible to macroscopically distinguish them from the different ducts; therefore, they were not measured (Fig. 11C and 11D). Testis colour was visually graded from 1 to 6, and different shades were observed during BS, varying from whitish to black, grey, and bicolored testes. The BS testes were graded as 1 and 2 for black or dark grey, respectively (Table III and Fig. 1).

Microscopic description: Histology and EM

Testes

The testicular capsule was composed of myoepithelial cells, connective tissue, and blood vessels, and differentiated into three layers: tunica serosa, tunica albuginea, and in the inner position, tunica vasculature (Fig. 12A and 12 B). Testicular capsule thickness was similar between BS and NBSs (Table IV). This capsule surrounded the seminiferous epithelium, which was composed of various segments of ST and interstitial space (Fig. 12 C and 12D). Inside the ST, spermatogenesis occurred, and all stages were observed from spermatogonia to spermatozoa and Sertoli cells (Fig. 12E and 12F). The ST diameter was smaller out of season (Table IV) and mostly early germ cells, such as spermatogonia and primary spermatocytes, and only a few sperm cells were found inside the tubules (Fig. 12F), whereas spermatids and mature spermatozoa were abundantly observed in samples harvested during the BS (Fig. 12F). The interstitial space contained blood vessels and, as predicted by the dark colour of the testes, melanocytes with melanin granules (Fig. 13A). In the TEM images, the composition of the interstitial space was clear, with visible nuclear and cytoplasmic components of melanocytes, Leydig cells, and blood vessels (Fig. 13B, 13C, and 13D). Testis pigmentation was related to the abundance of melanocytes/melanin granules visualised in the interstitial space, indicating that darker testes contained more melanin granules (Fig. 1).

Epididymis and deferent duct

Numerous cross-sections of the epididymal duct lumen were surrounded by smooth muscles, connective tissue, and a serosal outer layer (Fig. 14A and 14B). The lumen of the duct, the epididymal



mucosa, was covered with a simple cuboidal to pseudostratified columnar epithelium with ciliated cells (Fig. 14C and 14D). Inside the lumen, spermatozoa and round cells were found during the NBS and BSs; however, during the latter, spermatozoa were more abundant (Fig. 14C and 14D). In the SEM images, the ciliated epithelium of the epididymal lumen was observed in the presence of sperm cells and round cells (Fig. 14E and 14F). The deferent duct showed a single lumen surrounded by a thick layer of smooth muscle, connective tissue, and an outer serosal layer, and the mucosa was covered by a pseudostratified columnar epithelium (Fig. 15A and 15 B). A few spermatozoa were visible inside the lumen during breeding and NBSs.

Sperm morphology classification

Normal sperm morphology

Houbara spermatozoa presented a round-shaped acrosome, a long filiform nucleus similar to other non-passerine avian sperm, a midpiece, and a flagellum (Fig. 16A, 16 B, and 16C). When visualised by TEM, the acrosomal region did not present any *perforatorium* and *rostrum-like* structure and the nucleus contained mostly tightly compact chromatin (Fig. 16D). The midpiece contained a variable number of mitochondria, which could be visualised when the midpiece membrane was absent from the SEM images (Fig. 16C). Transverse sections of the midpiece showed 4–5 mitochondria around the axoneme, and 3–4 mitochondria could be counted along the midpiece length, resulting in 12–20 mitochondria per midpiece per sperm cell. The measurements for each sperm cell segment are presented in Tables Va and Vb. In the samples analysed, a mean of 68.5 % (± 14.7) of spermatozoa in the ejaculates (N=417) had a normal morphology (Table VI).

Acrosome defects

In the ejaculates analysed, 3.5 % (± 4.12) of sperm cells had an absent/detached acrosome. On phase-contrast microscopy and TEM, this defect was characterised by sperm cells completely missing the acrosome (Fig. 17A1, 17C, and 17D) or with a detached or partially detached acrosome that was still visible on the side of the nucleus (Fig. 17A2, 17E). In the SEM images, only complete loss of the acrosome was observed (Fig. 17B).

Midpiece

Midpiece defects were the most abundant morphological alterations (8.06 ± 7.19 %), which could be visualised as an abnormally disorganised shape or bent laterally to the head midpiece (Fig. 18A, 18B, and 18C). Disorganised mitochondrial distribution was observed in the TEM images when this alteration was present (Fig. 18D).



Cytoplasmic droplet

Few spermatozoa analysed, 2.08 % (± 1.89), had a structure similar to a large cytoplasmic droplet. In phase-contrast micrographs, the cytoplasmic content can be seen around the head (Fig. 19A1) or attached to the midpiece (Fig. 19A2). In the SEM images, the midpiece and part of the nucleus appeared swollen, and it was difficult to confirm if these images were related to this alteration (Fig. 19B). However, in the TEM micrographs, the cytoplasmic content is clearly visible and can be partially attached to the nucleus (Fig. 19C1) or fully surrounded, causing the midpiece to bend (Fig. 19C2). This defect is often associated with midpiece and tail abnormalities (Fig. 19A2 and 19C1).

Head defects

Several head alterations were observed. The first was underdeveloped heads, where phase contrast microscopy (Fig. 20A1) and SEM (Fig. 20A2) revealed that these sperm cells had a very small nucleus and seemed to lack a midpiece and acrosome. The second type of alteration was bent heads; on phase contrast microscopy (Fig. 20B1) and SEM (Fig. 20B2) photographs, these sperm cells appeared with a normal-sized nucleus, but bent or coiled. These two defects were grouped as head abnormalities, representing 5.35 % (± 4.27) of abnormal sperm morphology. A third type of head alteration was swollen heads, and phase contrast microscopy revealed that these sperm cells had a swollen nucleus that appeared darker and wider, but the acrosome and midpiece appeared normal (Fig. 20C1). In SEM images, the nuclei appeared wider (Fig. 20C2). This defect might be due to a DNA decompaction and represents 3.88 % (± 9.03) of sperm morphology defects.

Tail

Tail defects represent 4.57 % (± 5.22) of abnormal sperm morphology. On phase-contrast microscopy and SEM photographs, sperm cells were observed with coiled or bent tails (Fig. 21A1 and 21B1) or at the base of the midpiece (Fig. 21A3, 21B3, and 21C3), defibrillated (Fig. 21B1), and partly or completely absent (Fig. 21A2, 21C2).

Teratogenic

On average, 4.07 % (± 4.49) of sperm cells analysed presented teratogenic defects. Phase-contrast microscopy, SEM, and TEM revealed spermatozoa with double (sometimes triple) tails (Fig. 22A, 22 B, and 22C) and/or heads (Fig. 22D, 22E, and 22F).

Discussion

This study presents, for the first time, a description of the anatomy and histology of the reproductive tract of male and female North African houbara bustards during and outside the BS. Regression of the reproductive organs during the NBS was observed in both males and females.



Houbara males had larger ST diameters and testes, both in length and width, during the BS, compared with those in the NBS. More abundant advanced stages of spermatogenesis (spermatids and spermatozoa) were noted inside the ST in most testicular histological slides during the BS, suggesting spermatogenic activity and sperm production. These results are consistent with those of other studies on seasonal breeding birds that showed increased testicular weight, larger ST diameter, and high spermatogenic activity during the breeding period, such as the Japanese Quail (*Coturnix japonica*) (Artoni *et al.* 1999), African Penguin (Mafunda *et al.* 2021), Greater Rhea (Carvalho *et al.* 2015; Freneau *et al.* 2016), Red-winged Tinamou (*Rhyrchotus rufescens*) (Baraldi-Artoni *et al.* 2007), Jungle Crow (*Corvus macrorhynchos*) (Islam *et al.* 2010), Emu (Du Plessis 2012), Yangzhou Goose (*Anser cygnoides*) (Akhtar *et al.* 2020), and California Gull (*Larus californicus*) (Johnston 1956a). Avian testis size increases during the reproductive period in seasonal breeds (Møller 1988) and is correlated with ST diameter, germinative epithelium thickness, and sperm production. It should be noted that although houbara testes are less active outside of the BS, spermatogenesis does not seem to be completely stopped, as some late germ cells inside the STs and a few spermatozoa and round cells inside the epididymal lumen are still visible. During the BS, mature spermatozoa and round cells were more abundant inside the epididymal lumen. These round cells resembled round germ cells or sperm with large cytoplasmic droplets around the nucleus. This supports the results of previous studies suggesting that sperm maturation may occur during epididymal transit in houbara (Meunier *et al.* 2022). Meunier *et al.* (Meunier *et al.* 2022) showed that sperm quality improved over longer time intervals between ejaculate collections. Ejaculates collected at 5-day intervals had better concentrations, volumes, viability, motility, and morphology compared with those at 1-day intervals, suggesting that a longer transit time is beneficial for the completion of sperm development (Meunier *et al.* 2022). Bernal *et al.* also reported evidence of sperm maturation in the male duct, showing that sperm chromatin compaction increased through the vas deferens transit in the rooster (Bernal *et al.* 2022).

Regression of the ovary and oviduct was visually clear, although it was not statistically analysed because of the small sample size during NBS. However, the oviduct of the two females out of the BS was 4.8 to 6.5 times smaller. During the BS, two to three pre-ovulatory follicles are observed, whereas during the NBS, only pre-hierarchical follicles are visible. This regression of the oviduct and ovary is similar to that observed in other seasonal breeders, such as California gulls (Johnston 1956b) and African penguins (Mafunda *et al.* 2021).

This regression of the reproductive organs is consistent with the hormonal pattern previously reported for houbara bustards. During the BS, luteinizing hormone (LH) and progesterone levels in females and LH and testosterone levels in males were higher (Saint Jalme *et al.* 1996), accounting for



differences in spermatogenesis and folliculogenesis during the BS and NBS. In seasonal females, light influences the functions of gonadotropin-releasing hormone, LH, follicle-stimulating hormone (FSH), and prolactin. These hormones play a crucial role in regulating the reproductive seasonality of both long- and short-day breeders by affecting their gonadal function (Sharp & Blache 2003).

Adequate FSH and LH levels can promote follicular growth and lead to egg production by facilitating follicular development and ovulation. FSH primarily initiates follicular growth, whereas LH targets preovulatory follicles. The synergistic action of FSH and LH is essential for normal follicular development. Insufficient levels of FSH and LH lead to follicle formation, but retard egg production in chickens (Parizzi *et al.* 2008).

Most bird species present testicular asymmetry between the left and right testes in terms of shape, size, and weight, in favour of the left side (Briskie, J. V. & Montgomerie, R. 2007; Aire 2007). One possible explanation for this phenomenon is that the position of certain organs, such as the liver, is also asymmetrical, and therefore, it would limit the space for testicular growth on the right side, making the left side bigger (Calhim & Birkhead 2009). However, our results showed symmetry in the shape and size of the right and left testes.

Overall, the anatomy and histology of the male and female reproductive tracts were similar to those of other avian species, such as chickens (Razi *et al.* 2010; Mohammadpour *et al.* 2012; Henna Wani, Mohammad Maqbool Darzi, Shayaib Ahmad Kamil, Shakil 2017; Apperson *et al.* 2017), turkeys (Mohamed Nabil *et al.* 2022), domestic ducks (*Ansa ansa domesticus* and *Anas boschas domesticus*), (Mohammadpour *et al.* 2012; Essam, M. EL Gendy, Asmaa M. I., Samah H. El-Bably, Nora A. Shaker, Shaimaa H.H. 2016; H. Mahmoud *et al.* 2018) and greater rheas (Carvalho *et al.* 2015; Freneau *et al.* 2016). However, anatomical and histological analyses revealed that houbara contains melanin pigments in the testes, ovaries, and some oviduct segments because of the presence of melanocytes. This pigmentation was present during both the BS and NBS. This pigmentation is explained by the presence of melanin in the capsule and interstitial tissue and has been previously reported in some avian species, such as in the Rhea and Ostrich, and cassowary (*Casuaris casuaris*), where they are tan-coloured (Murray E. Fowler 1991; Soley JT 1992) in the Emu they are black (Du Plessis 2012) and grey to black in cockatoos, mynahs, and toucans (Taylor M. 1989). The majority of domestic avian species have colourless testes.

Melanin has also been observed in the ovaries and oviducts of some silky (Chen *et al.* 2008) and Nigerian fowls (Aire & Steinbach 1973). However, its function in the avian reproductive tract remains unclear. Pigmented testes were reported (Agrawal & Bansal 1983) only during the NBS in the Indian robin, and these pigmented testes showed reduced spermatogenic activity, prompting the



authors to suggest that testis melanisation might be due to adrenal gland dysfunction that affects spermatogenesis. Galván *et al.* (2011) found that testis melanisation in birds was positively correlated with mtDNA mutations, suggesting an adaptive function linked to protection against oxidative stress. Melanin and its possible functions have been studied extensively in non-avian species. In bats, melanin is found around the testes and epididymis, suggesting that it most likely protects mature sperm against UV radiation and oxidative stress during storage inside male ducts (Delmore *et al.* 2018). Our previous findings showed an increase in the intracellular oxidative stress marker (DHE) when the sperm collection interval was extended to 10 days, compared to 1 and 5 days (Meunier *et al.* 2022). In frogs, melanin protects thermosensitive organs (Franco-Belussi *et al.* 2017) and exposure to UVR causes an increase in internal melanin pigmentation (Franco-Belussi *et al.* 2016). In the Mexican Lizard (*Sceloporus variabilis*), the testis pigmentation (inside the tunica albuginea) is unilateral, only the active testis being coloured; the correlation between melanin pigmentation and spermatogenic activity and ST diameter also suggests a possible role in the thermal physiology of this species (Guillette *et al.* 1983). Collectively, these results suggest that melanin inside the testes may protect spermatozoa against oxidative damage as sperm cells are prone to oxidative stress due to their reduced cytoplasm and lack of DNA repair system (Velando *et al.* 2008). This hypothesis could also be considered for the melanin observed in the oviduct, especially in sperm storage sites (UVJ), as sperm cells can be stored for weeks before fertilisation occurs, and also in the ovaries around the follicles, as female gametes can also be prone to oxidation, and the embryonic origin of the tissues is the same.

Our results showed that houbara sperm is shorter than in most avian species with an average length of 46 μm . Avian sperm cells have a wide range of size, from 30 to 300- μm (McFarlane 1963). The midpiece length (average size of 1.88 μm) and number of mitochondria (12 to 20) are notably low compared with those of other avian species. The mitochondrial number in avian sperm is highly variable across species, ranging from 6 to 12 in the White Pekin Duck (*Anas platyrhynchos*) (Majhi *et al.* 2016) to 1400 in the Japanese Quail (Korn *et al.* 2000); however, for most studied species it is above 20. Sperm length, and especially midpiece length, are believed to be correlated with sperm velocity and contribute to sperm competition, as faster swimming spermatozoa would have a better chance of fertilising an egg when competing with sperm from other males (Fitzpatrick *et al.* 2009; Kleven *et al.* 2009; Bennison *et al.* 2015). However, the relationship between sperm length velocity and sperm competition is not always clear, (Humphries *et al.* 2008; Simmons & Fitzpatrick 2012) and it is important to highlight that after ejaculation, sperm must reach SSTs, where they remain immotile during storage and regain motility when recruited for fertilisation. Therefore, the motility to reach the storage site and later fertilisation might be different. Although little is known about the underlying reactivation mechanisms, mitochondrial metabolism may play a key role. The midpiece length and



number of mitochondria may reflect the amount of energy needed for movement (Korn *et al.* 2000). Therefore, sperm with longer midpieces would require more energy. Gu *et al.* showed that mitochondrial volume was positively correlated with ATP production and sperm velocity in 10 mammalian species (Gu *et al.* 2019). There are two main metabolic sources of energy in avian sperm: aerobic respiration and anaerobic glycolysis (Du Plessis *et al.* 2015). Previous analysis of houbara sperm (unpublished data) showed a loss of motility (close to 0 %) when the mitochondrial respiratory chain was inhibited, suggesting that houbara sperm relied mainly on aerobic respiration. This metabolism makes sperm prone to oxidative stress by producing reactive oxygen species, especially during long storage periods inside the oviduct. It is possible that the relatively low number of mitochondria in houbara sperm limits oxidation and protects sperm integrity during storage. The mitochondrial number may also be explained by a possible reduced energy requirement due to short sperm length. The number of mitochondria is believed to be linked to the phylogenetic position, according to Fawcett (1970). However, this was difficult to verify, as we did not have any other data on sperm morphology in Otidiformes.

The sperm morphology of houbara birds is similar to that of other non-passerine birds. Although a noticeable difference was found, houbara sperm does not have a perforatorium; the acrosome has a round shape and is directly attached to the anterior side of the nucleus, which is completely flat because the nuclear rostrum is also absent, similar to the Red-bellied Woodpecker (*Melanerpes carolinus*)(Henley *et al.* 1978), Jacana (*Jacana jacana*)(Saita *et al.* 1983) and Cattle Egret (*Bubulcus ibis*)(Roopnarine *et al.* 2020).

Houbara sperm presented 32 % of abnormal morphology and the most common abnormalities were midpiece (8 %) and head defects (5 %). In a previous study, no difference was found in the percentage of morphologically normal sperm between old (n= 111) and young (n= 110) houbara bustards (Meunier *et al.* 2022), and the means were similar to those in this study (for abnormal, 40.44 ± 14.96 % for old and 32.17 ± 9.51 % for young males, data not published). This proportion of defects is higher compared to what was observed for other avian species documented in the literature: the percentage of abnormal sperm ranges from 17 % in turkeys (Alkan *et al.* 2002) to 25 % in the Rock Pigeon (*Columba livia*)(Sontakke *et al.* 2004), and main defects are midpiece, acrosome, and tail abnormalities. However, as classification differs among species and no sperm morphology of birds of the same order has been previously reported, it is difficult to conclude these differences.

Conclusion

In conclusion, the anatomy and histology of the reproductive tracts during the breeding and NBSs and sperm ultrastructure, morphometry, and morphology were described in both male and



female North African houbara bustards. Our results showed regression of both male and female reproductive tracts during the NBS. Although our anatomy, histology, and morphology results were similar to those of other avian species, some noticeable differences were found, such as symmetrical testes; the melanin pigment found inside the testes, ovary, and oviduct; and the absence of perforatorium and rostrum.

To our knowledge, this is the first detailed description of the reproductive anatomy, histology, and sperm morphology in avian species of the order Otidiformes. This order included 26 species, 15 of which are red-listed (2016). Despite *in situ* conservation efforts, populations continue to decline, and some bustard species are facing extinction risk. Ex situ initiatives (conservation breeding) represent a basic form of conservation insurance and simpler steps to buy time, while larger issues of population restoration and protection are addressed in situ (Collar & Wirth 2022). Conservation breeding programs are now being initiated for two critically endangered bustard species: the Great Indian Bustard (*Ardeotis nigriceps*) (Srinivas *et al.* 2022) and the Lesser Florican (*Sypheotides indicus*) (Collar 2021). Therefore, this study may serve as a model for identifying bustards and other endangered wild bird species. Further work on sperm storage inside females will provide more insights into insemination timing. Further investigation of melanin function and sperm maturation in the male duct would also be of interest.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

LM, YH, MSJ, GS, and JTC conceived the study; LM, CSV, and JTC collected the data, LM and JTC analysed the data and prepared the tables and figures; LM and JTC wrote the first draft of the manuscript; all authors edited, revised, and approved the final version.

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Figures

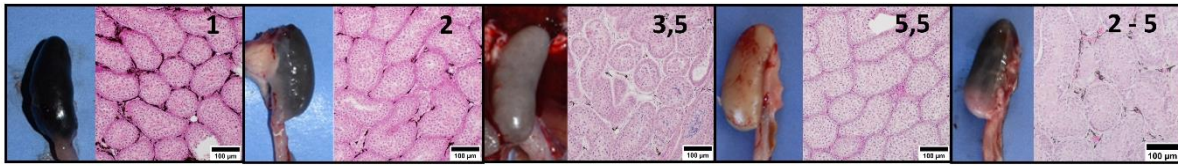


Figure 1: Testicular colour grade correlation with melanin pigment – Anatomy picture (on the right) of the testis with colour grade and corresponding light micrograph of the testis (hematoxylin and eosin staining - HE, x200).

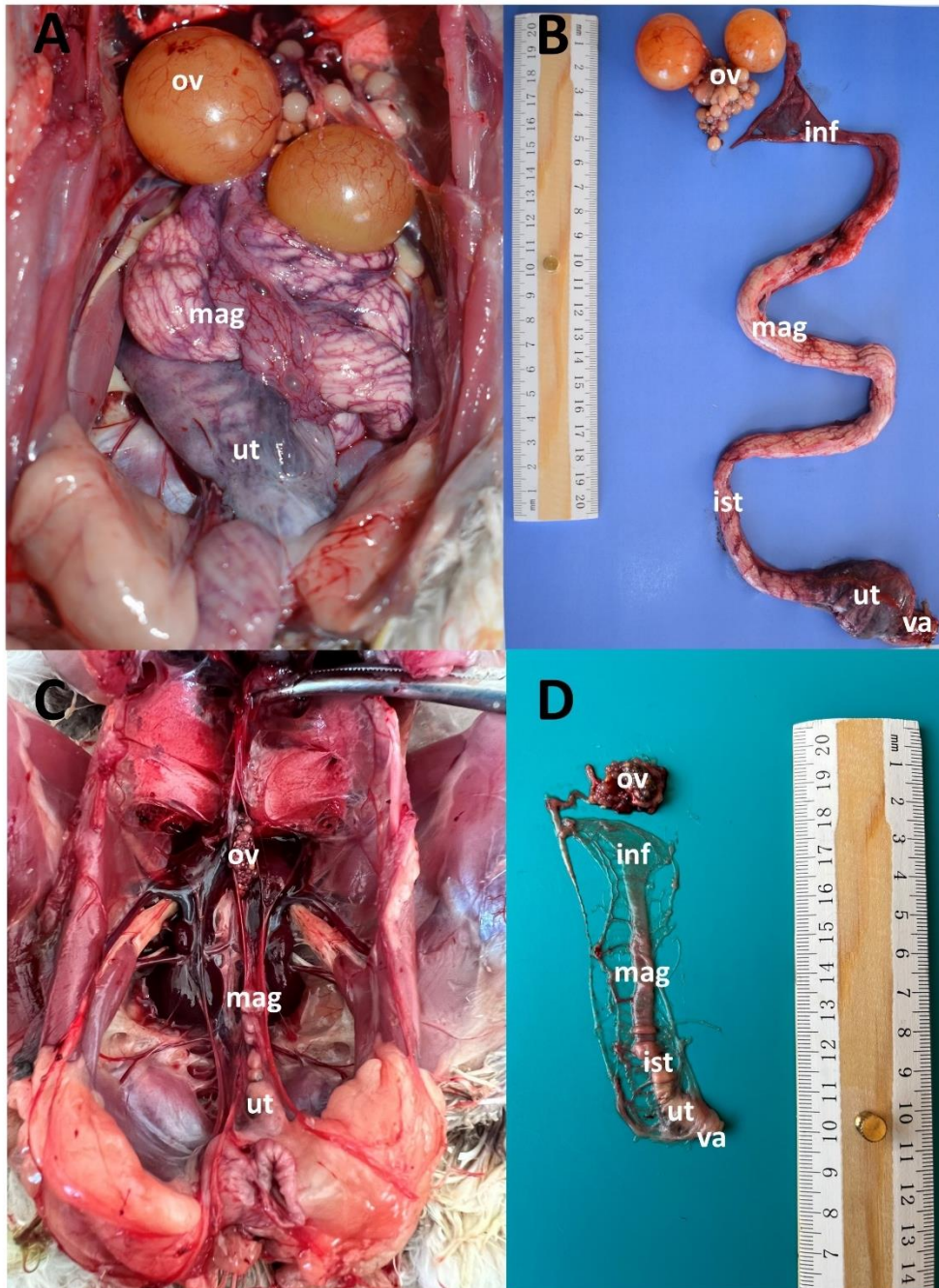


Figure 2: Anatomy of the North African Houbara Bustard female's reproductive tract during breeding season inside (A) and outside (B) the coelomic cavity and during non-breeding season inside (C) and outside (D) the coelomic cavity; Ovary (ov), infundibulum (inf), magnum (mag), isthmus (ist), uterus (ut), vagina (va).

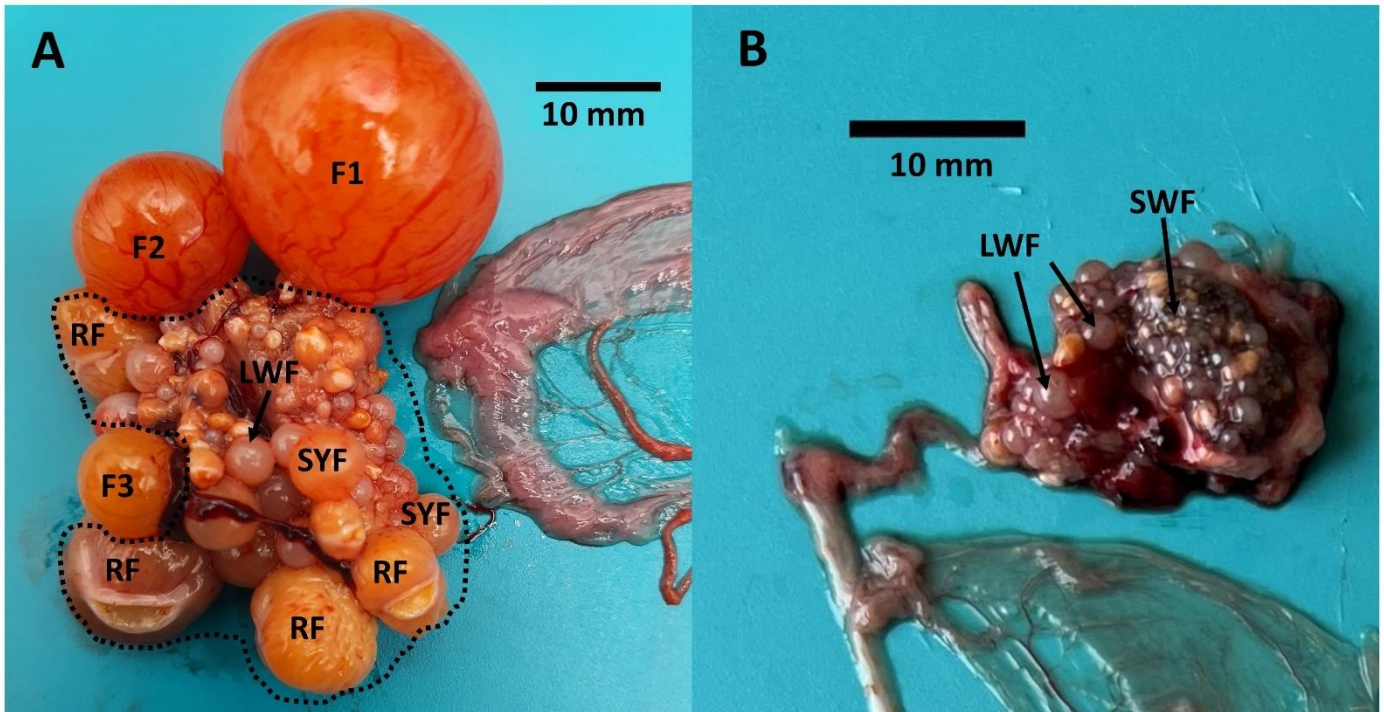


Figure 3 : Left ovary of a North African bustard female during the breeding season (A) with prehierarchal follicles surrounded by the dotted line ; large white follicles (LWF), small yellow follicles (SYF) and regressing post ovulatory follicles (RF) were visible; F1 to F3 correspond to hierarchal follicles (pre-ovulatory), F3 is the most recently selected follicle; **Left ovary of a North African bustard female during the non-breeding season (B)** :Only pre-hierarchical small white follicles (SWF) and large white follicles (LWF) are visible.

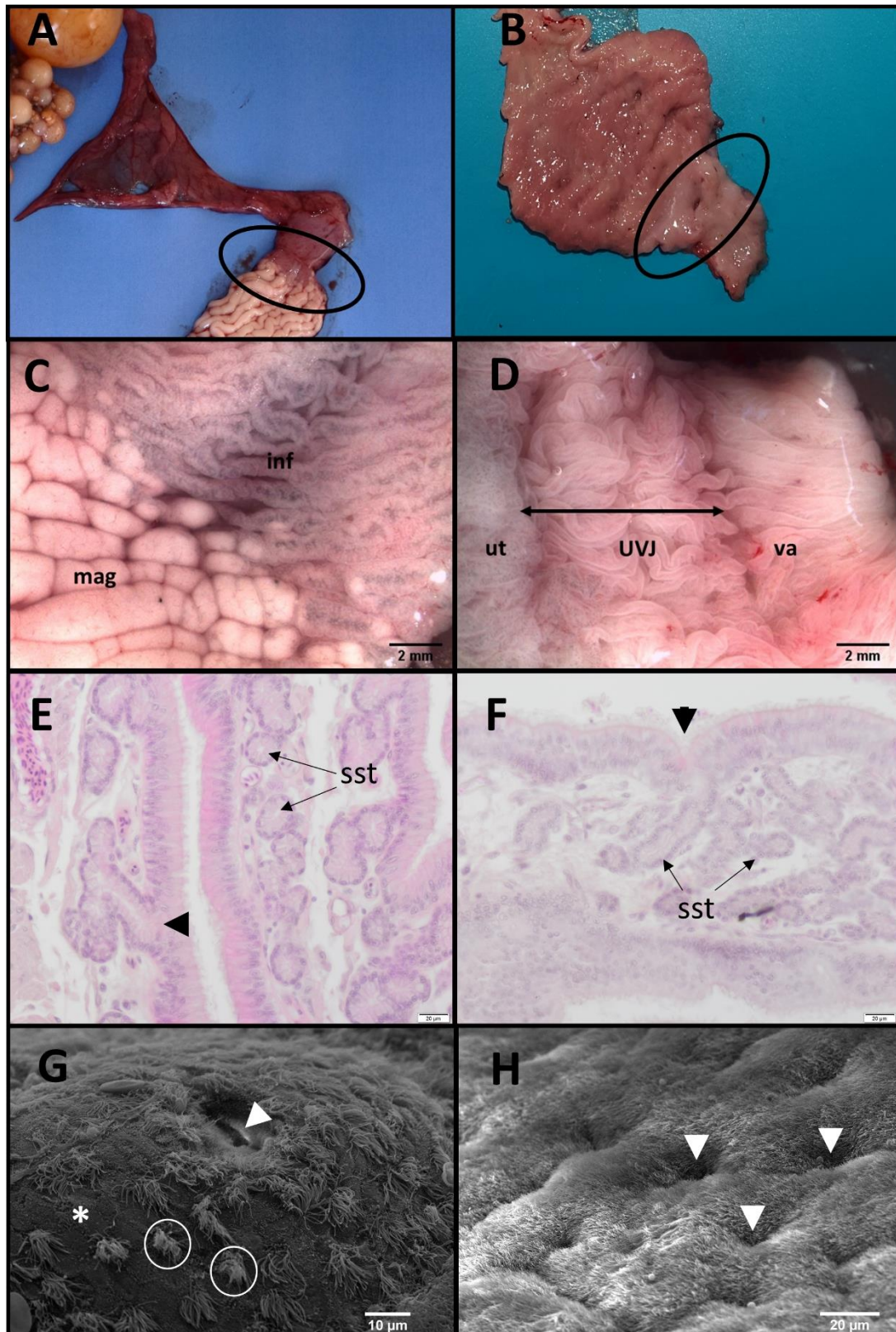


Figure 4: Anatomical location of the sperm storage tubules (SST) in the infundibulum (A) and in the utero vaginal junction (UVJ) (B), infundibulum (C) and UVJ (D) mucosa with visible folds containing SST (stereomicroscope observation), light micrograph of infundibulum (E) and UVJ (F) mucosa (HE, x400), black arrows showing cross section of SST's and black arrow head showing the entrance of SST, SEM



photograph of the infundibulum (G) and UVJ (H) mucosa, with arrow heads showing the entrance of SST, ciliated epithelium cells are circled in white and non-ciliated epithelium cells are indicated with a white asterisk.

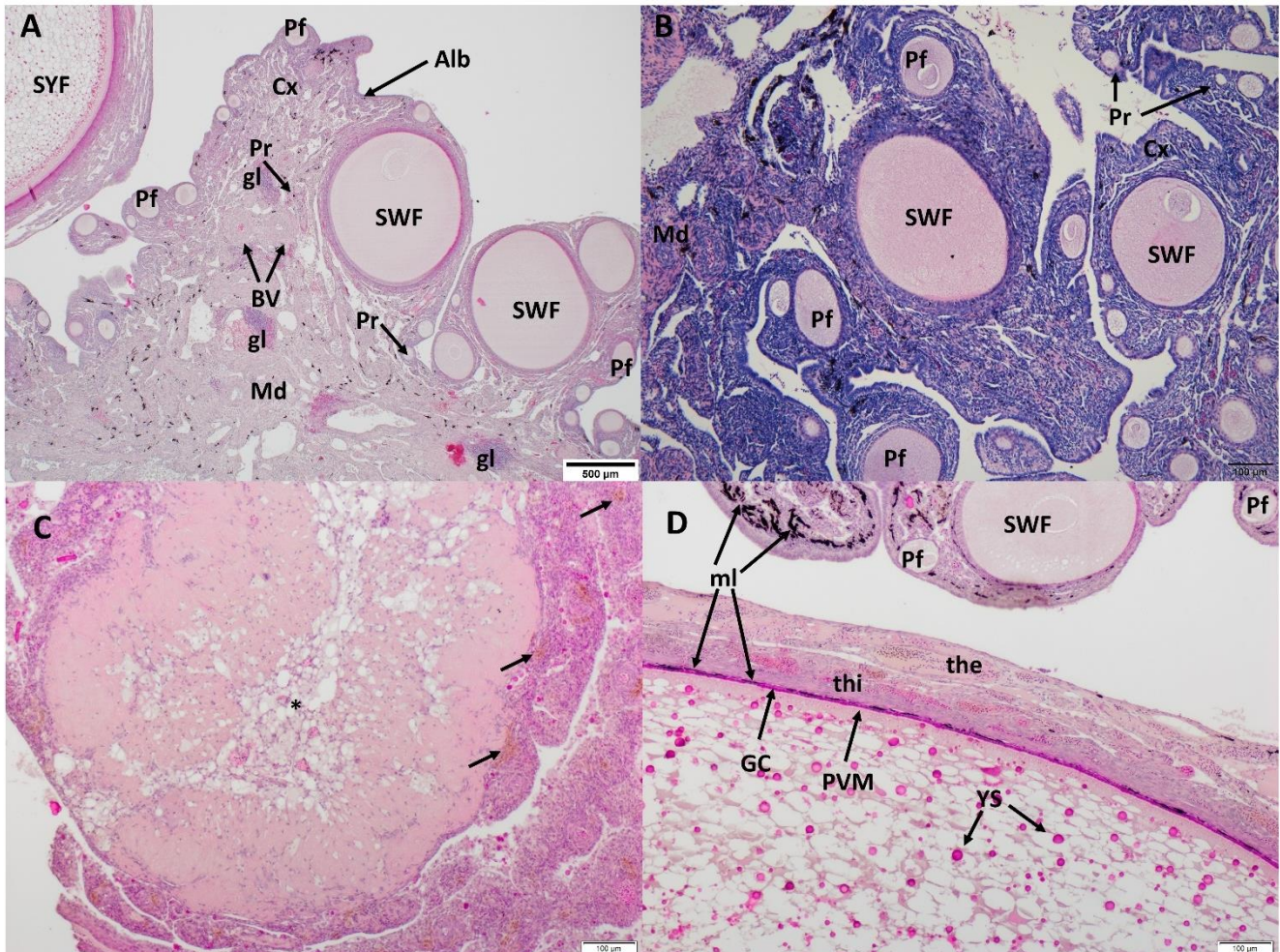


Figure 5: Histological micrographs of the ovary of the North African Houbara Bustard during breeding season (A) (HE, x400) and non-breeding season (B) (HE, x100); Albuginea (Alb), cortex (Cx), medulla (Md), blood vessel (BV), ganglion (gl), primordial follicle (Pr), primary follicle (Pf), small white follicle (SWF), small yellow follicle (SYF). **Histological micrograph of an atretic follicle (C)** (HE, x100); asterisk is showing vacuolated cells with lipidic inclusions and black arrows are indicating macrophages containing brown hemosiderin content; **Histological micrograph of a pre-ovulatory follicle (D)** (HE, x100); ; primary follicle (Pf), small white follicle (SWF), granulosa cells (GC), peri vitelline membrane (PVM), theca interna (thi), theca externa (the), yolk sphere (YS), melanin granules (ml), ovarian cuboidal epithelium (Ep).

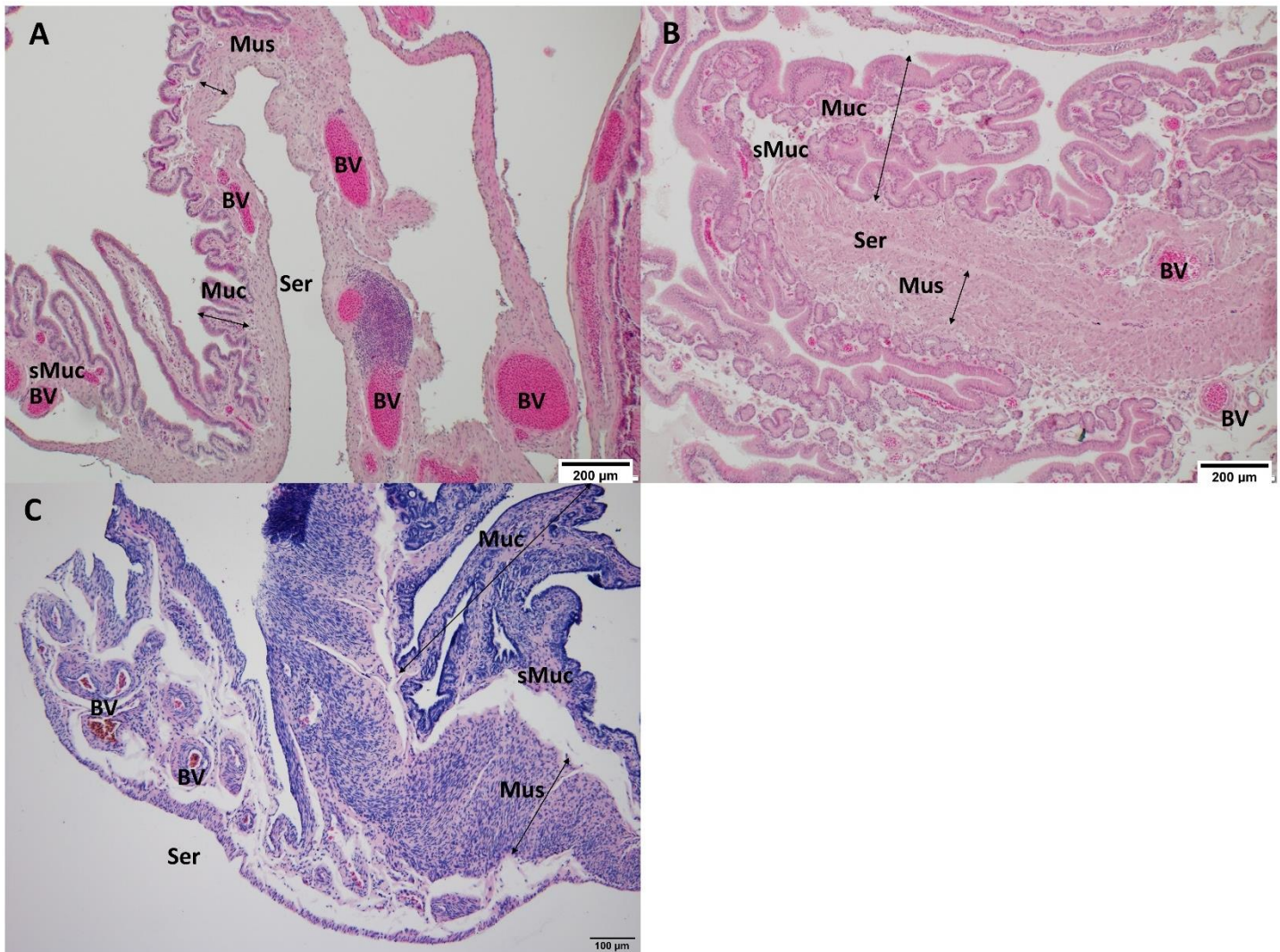


Figure 6: Histological micrographs of the infundibulum of the North African Houbara Bustard, funnel (A) and neck region (B) during breeding season; Histological micrographs of the neck region of infundibulum during non-breeding season (C) (HE, x100); Mucosa (Muc), submucosa (sMuc), muscularis (Mus), serosa (Ser), blood vessel (BV).

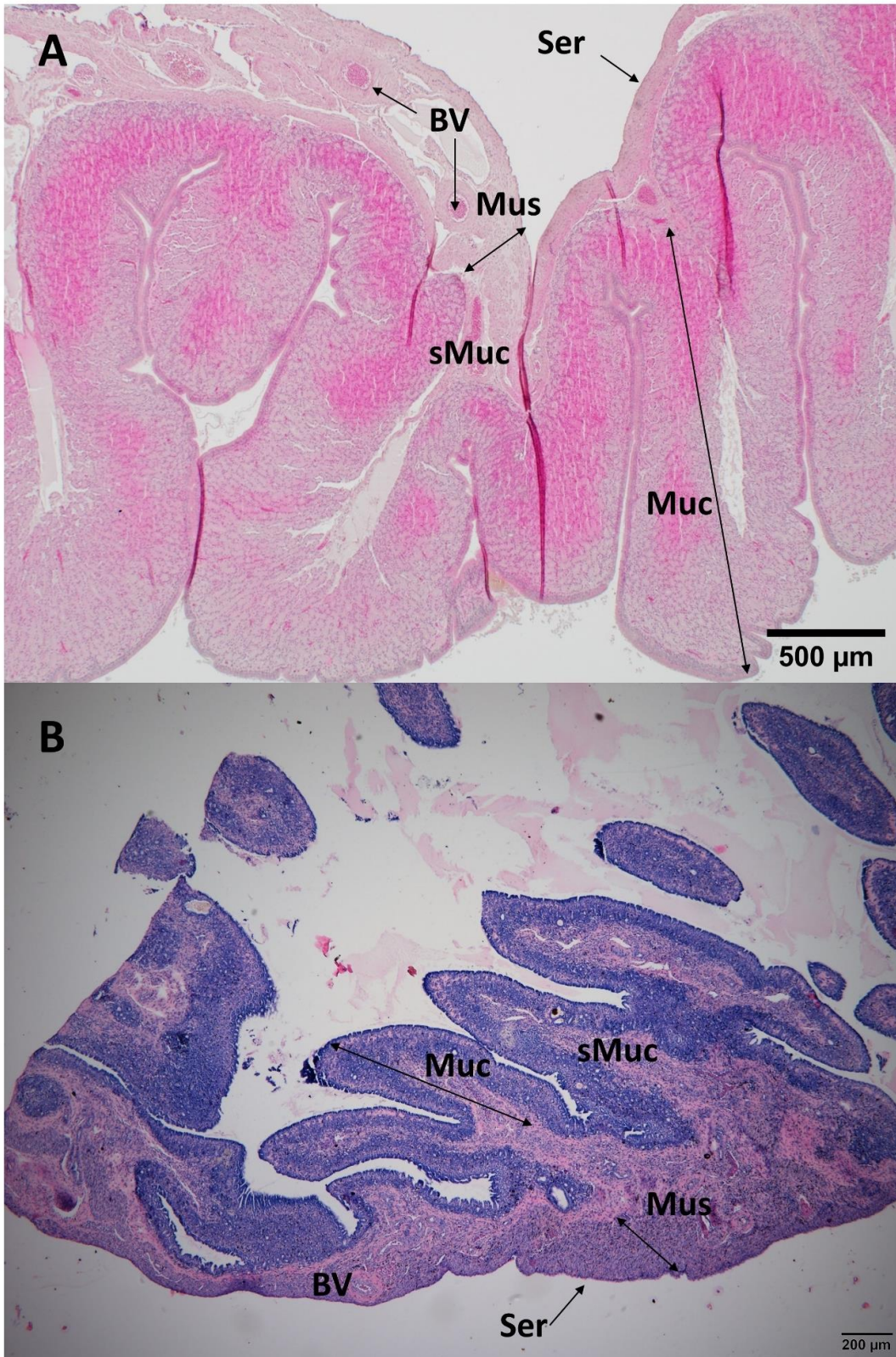


Figure 7: Histological micrographs of the magnum of the North African Houbara Bustard during the breeding season (A) and the non-breeding season (B) (HE, x40); Mucosa (Muc), submucosa (sMuc), muscularis (Mus), serosa (Ser), blood vessel (BV).

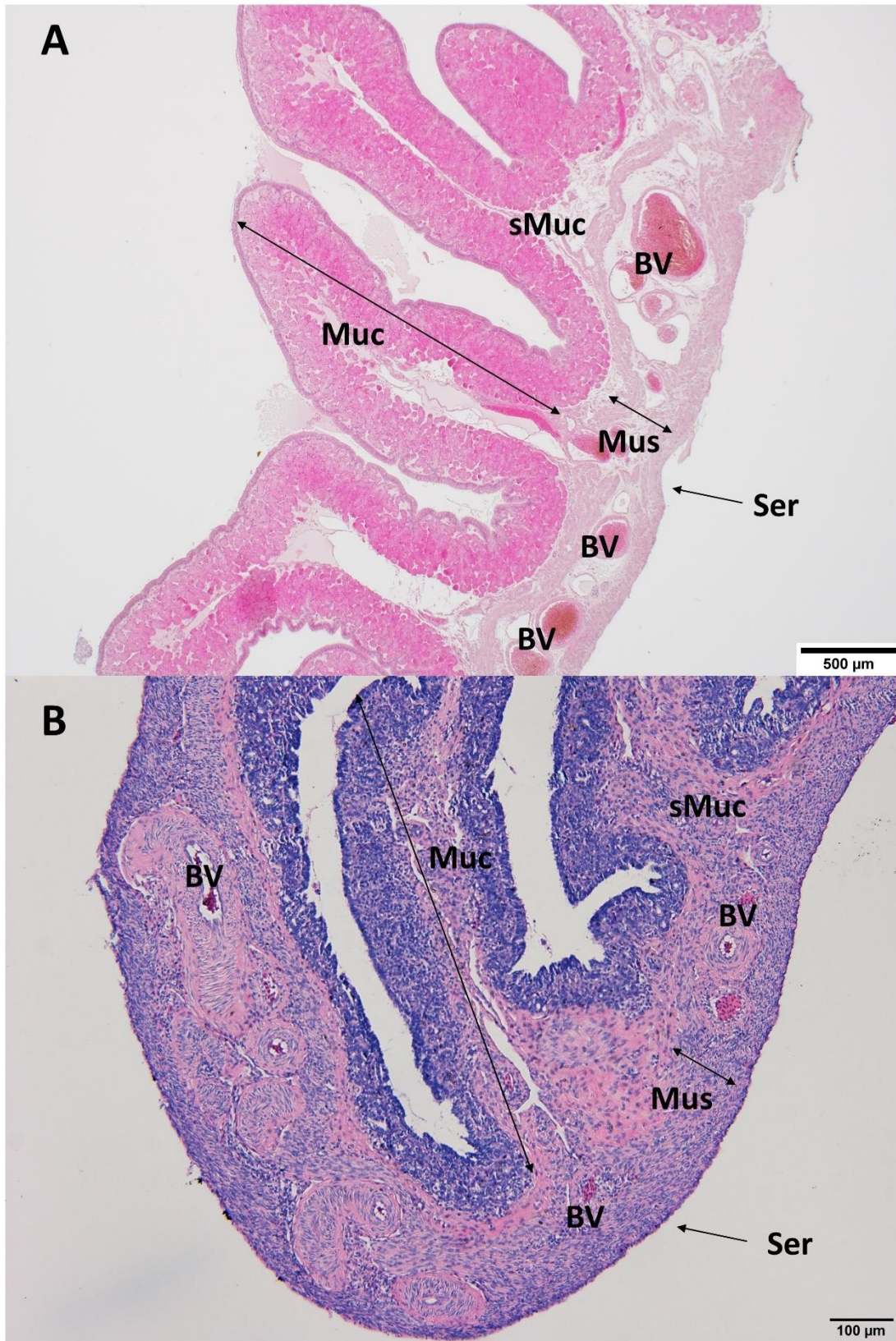


Figure 8: Histological micrographs of the isthmus of the North African Houbara Bustard during the breeding season (A) and the non-breeding season (B) (HE, x40); Mucosa (Muc), submucosa (sMuc), muscularis (Mus), serosa (Ser), blood vessel (BV).

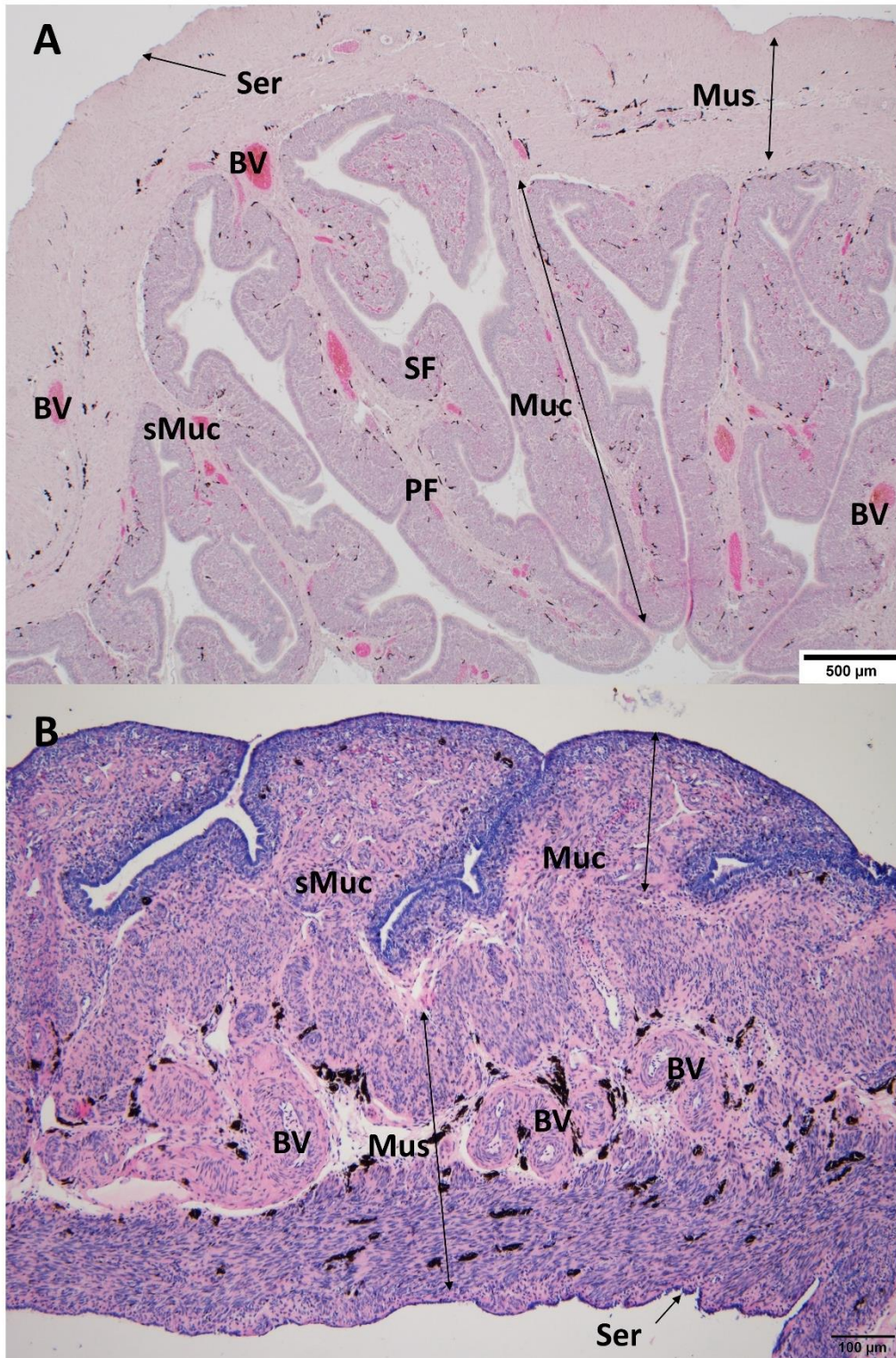


Figure 9: Histological micrographs of the uterus of the North African Houbara Bustard during the breeding season (A) and the non-breeding season (B) (HE, x40); Mucosa (Muc), submucosa (sMuc), muscularis (Mus), serosa (Ser), blood vessel (BV), primary fold (PF), secondary fold (SF); Melanin granules are visible in the mucosa, submucosa and muscularis tunica.

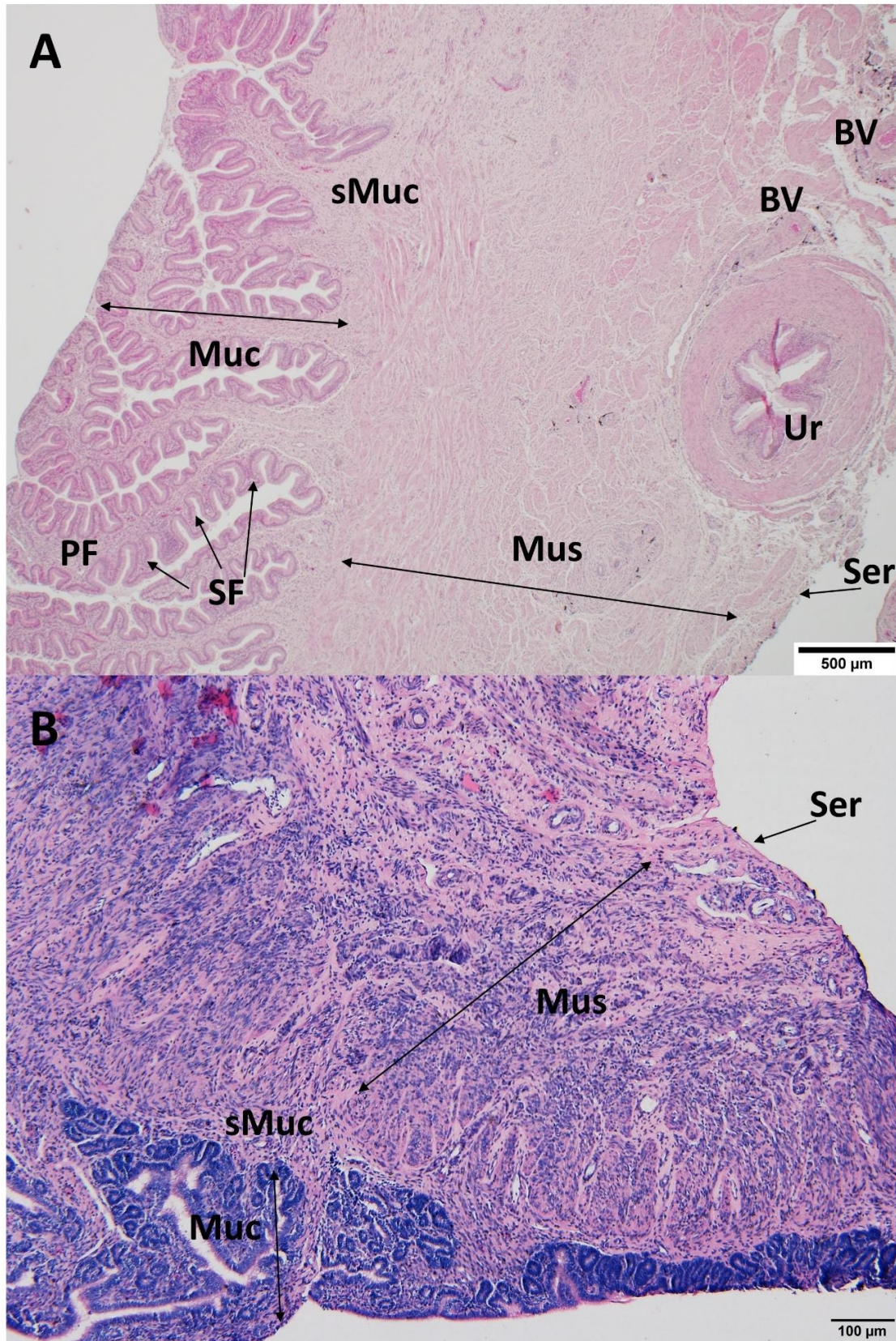


Figure 10: Histological micrographs of the uterus of the North African Houbara Bustard during the breeding season (A) (HE, x40) and the non-breeding season (B) (HE, x100); Mucosa (Muc), submucosa (sMuc), muscularis (Mus), serosa (Ser), blood vessel (BV), primary fold (PF), secondary fold (SF).

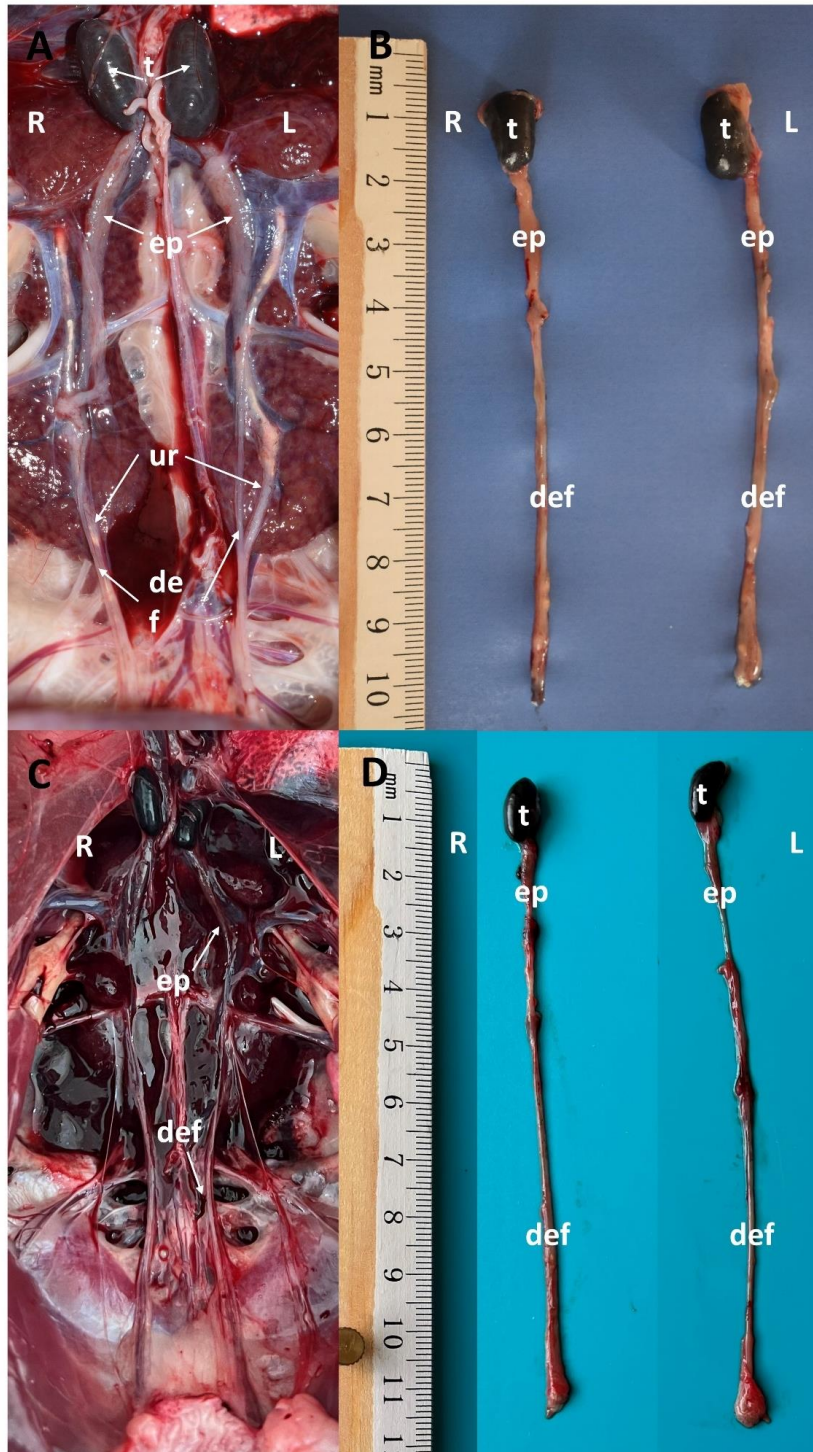


Figure 11: Anatomy of the North African Houbara Bustard male's reproductive tract during breeding season inside (A) and outside (B) the coelomic cavity and during non-breeding season inside (C) and outside (D) the coelomic cavity; Right (R) and left (L) side, testis (t), epididymis (ep), deferent duct (def), ureter (ur).

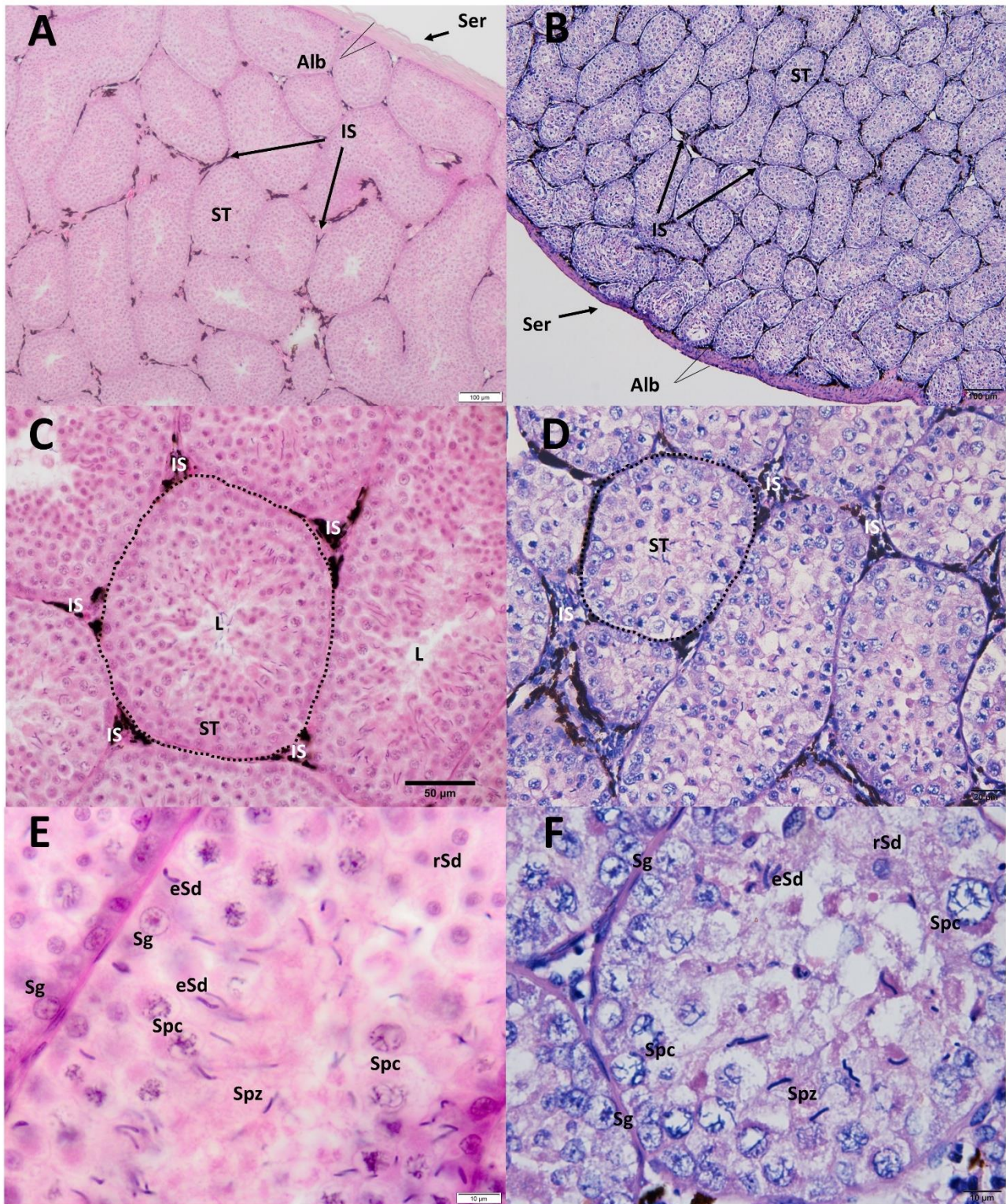


Figure 12: Histological micrographs of the testis of the North African Houbara Bustard during the breeding season x100 (A), x400 (C) and x1000 (F) and the non-breeding season x100 (B), x400 (D) and x1000 (G) (HE); Tunica albuginea (Alb), tunica serosa (ser), seminiferous tubule (ST), interstitial space (IS), lumen of the tubule (lu), spermatogonia (Sg), spermatocyte (Spc), round spermatid (rSd), elongated spermatid (eSd), spermatozoa (spz).

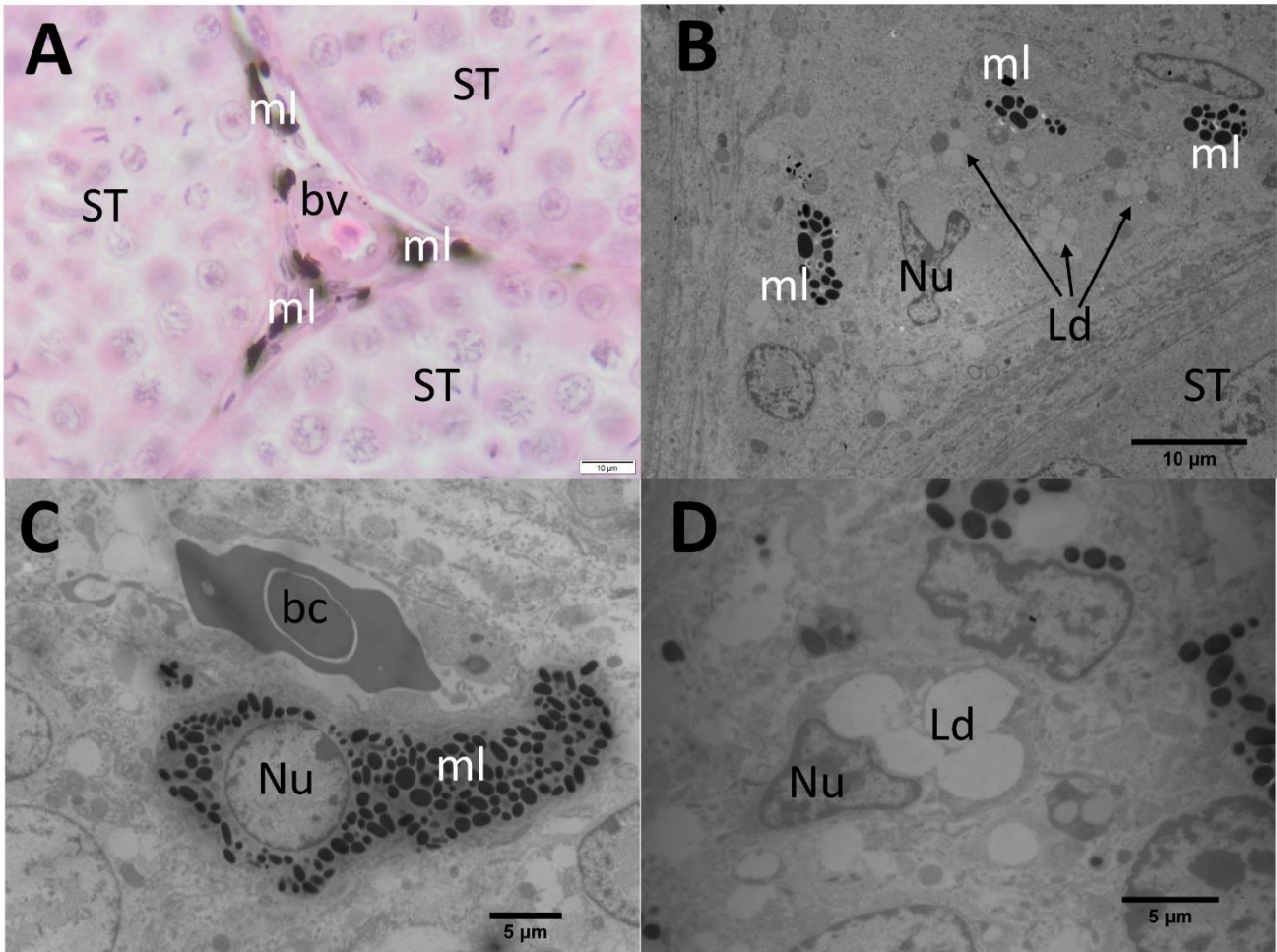


Figure 13: Histological micrograph of the interstitial space (A), (HE, oil immersion, x1000) and TEM photograph showing melanocytes (B, C) and Leydig cell (D). Seminiferous tubule (ST), melanin granule (ml), blood vessel (bv), nucleus (Nu), blood cell (bc), lipid droplets (Ld).

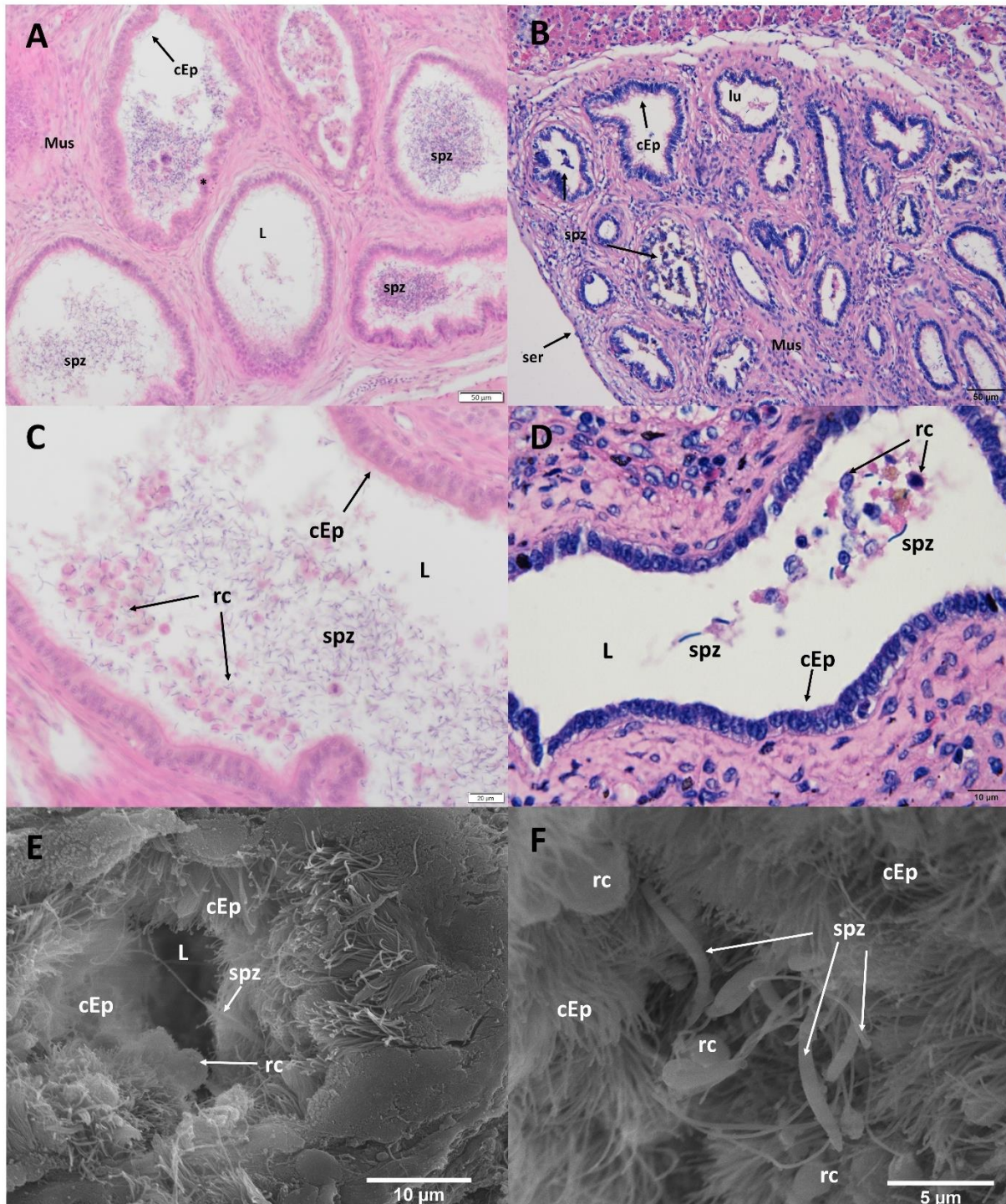


Figure 14: Histological micrographs of the epididymis of the North African Houbara Bustard during the breeding season (A) (HE, x200) and the non-breeding season (B) (HE, x200); Histological micrographs of the lumen of the epididymis duct during breeding season (C) (HE, x400) and the non-breeding season (D) (HE, x1000); SEM photographs of the lumen of the epididymis duct (E, F); Ciliated epithelium cell (cEp), muscularis (Mus), serosa (Ser), lumen (L), round cell (rc), spermatozoa (spz).

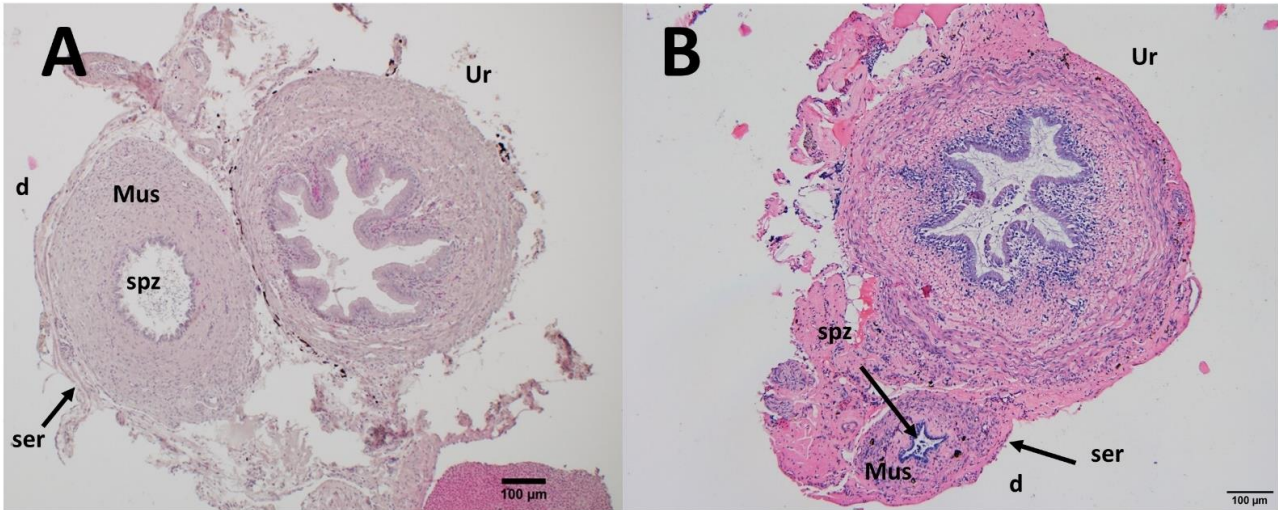


Figure 15: Histological micrographs of the deferent duct of the North African Houbara Bustard during the breeding season (A) and the non-breeding season (B) (HE, x100); Deferent duct (d), ureter (Ur), muscularis (Mus), serosa (Ser), spermatozoa (spz).

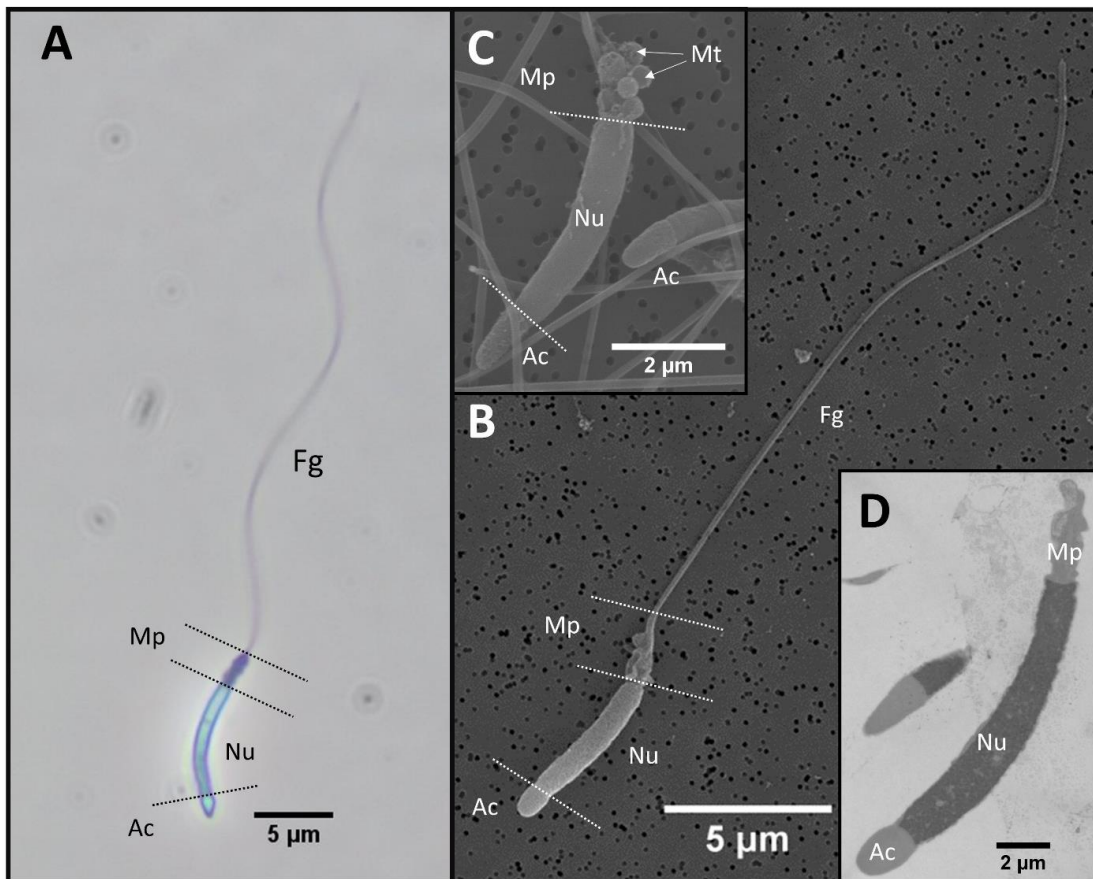


Figure 16: Normal sperm morphology of the North African Houbara Bustard observed with phase contrast microscopy (oil immersion, x1000) (A), SEM (B, C) and TEM (D) – Acrosome (Ac), nucleus (Nu), midpiece (Mp), mitochondria (Mt), flagellum (Fg).

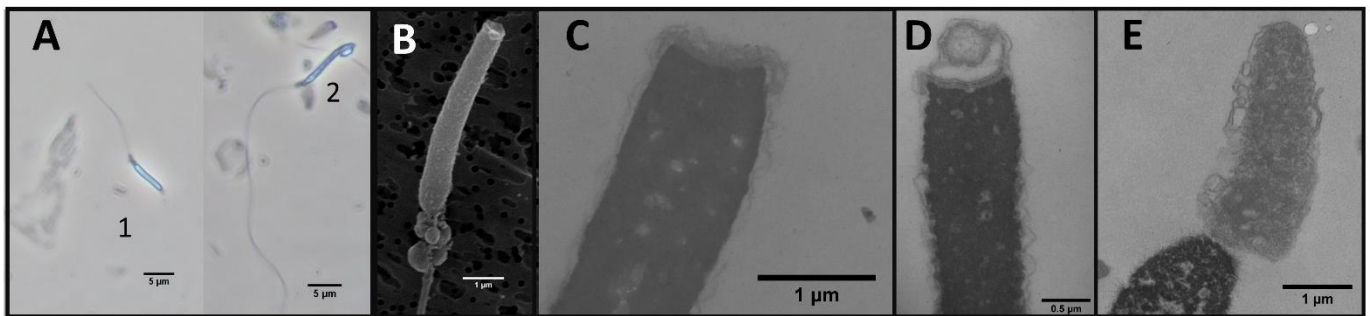


Figure 17: Acrosome defects of North African houbara bustards' sperm observed with contrast phase microscopy (oil immersion, x1000) (A), SEM (B) and TEM (C, D, E) – 1: sperm without acrosome, 2: sperm losing the acrosome.

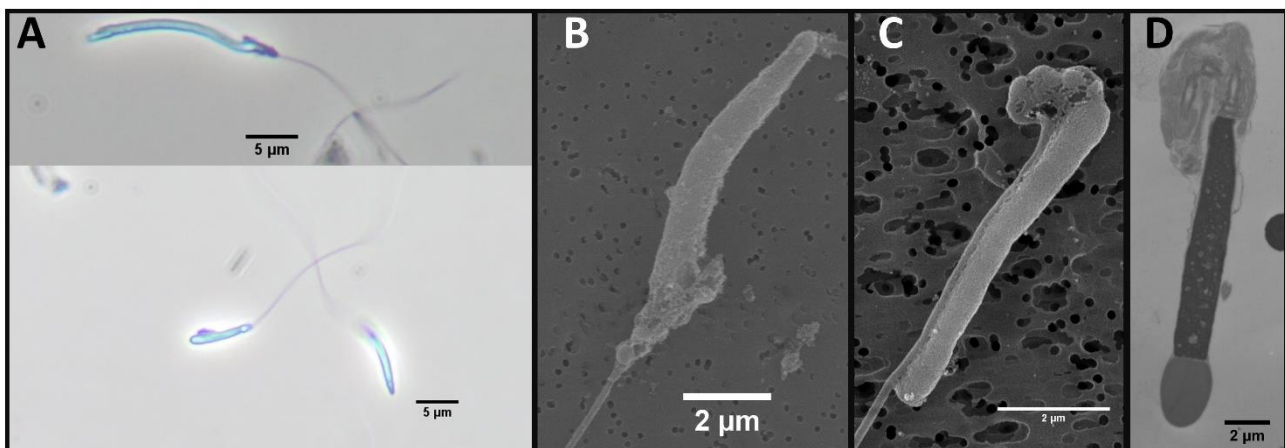


Figure 18: Midpiece abnormalities of North African houbara bustards' sperm observed with contrast phase microscopy (oil immersion, x1000) (A), SEM (B, C) and TEM (D).

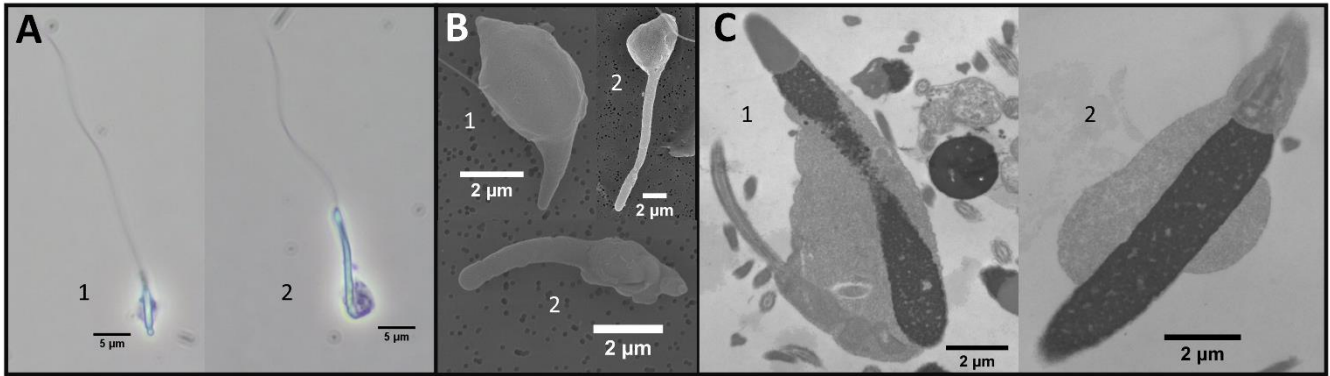


Figure 19: Cytoplasmic droplets of North African houbara bustards' sperm observed with contrast phase microscopy (oil immersion, x1000) (A), SEM (B) and TEM (C). 1: cytoplasmic droplet all around the head, 2: cytoplasmic droplet located around the midpiece and the basal part of the head.

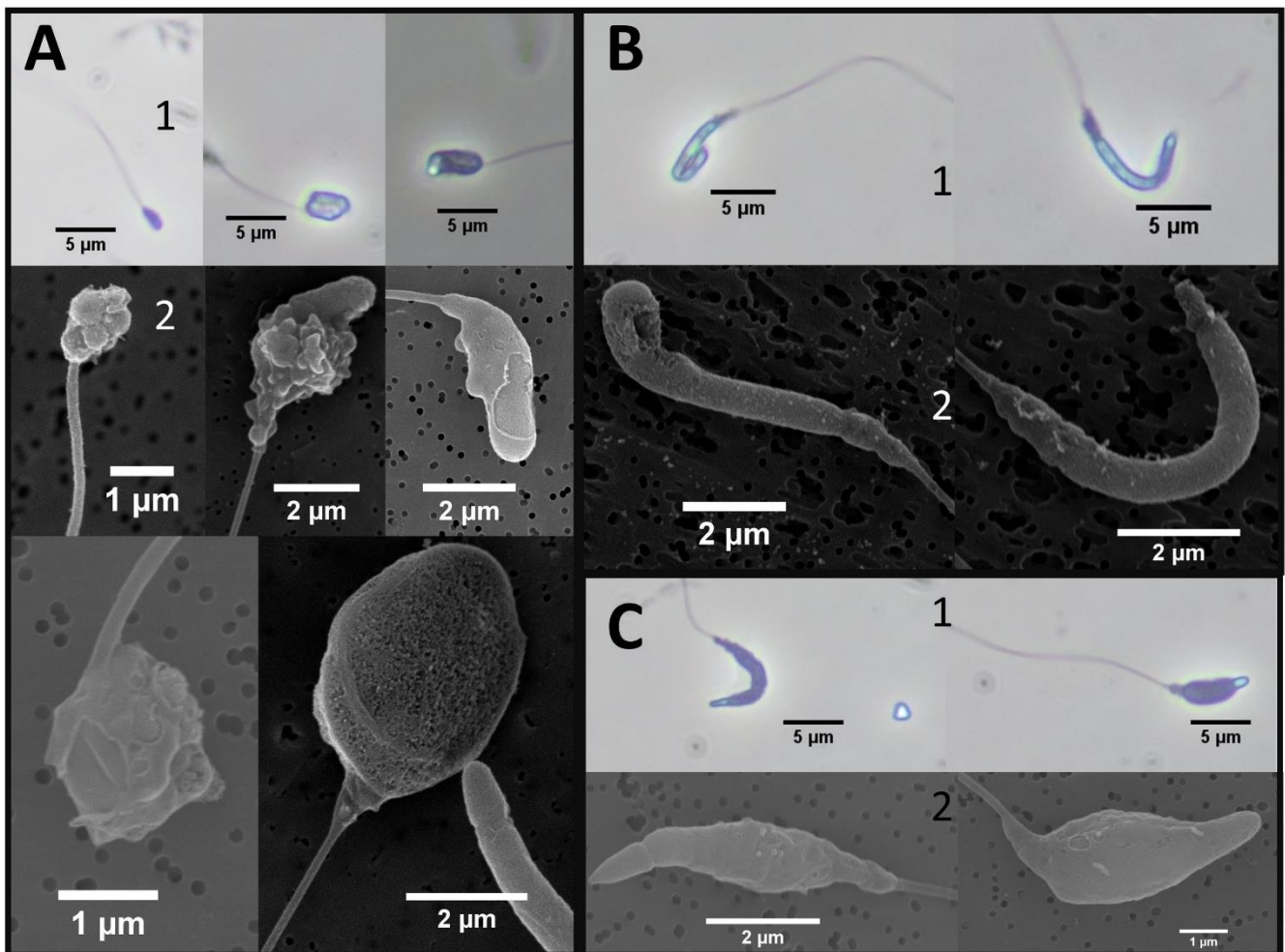


Figure 20: Head defects of North African houbara bustards' sperm: Underdeveloped head (A), bended head (B) and swollen head (C) observed with contrast phase microscopy (oil immersion, x1000) (1) and SEM (2).

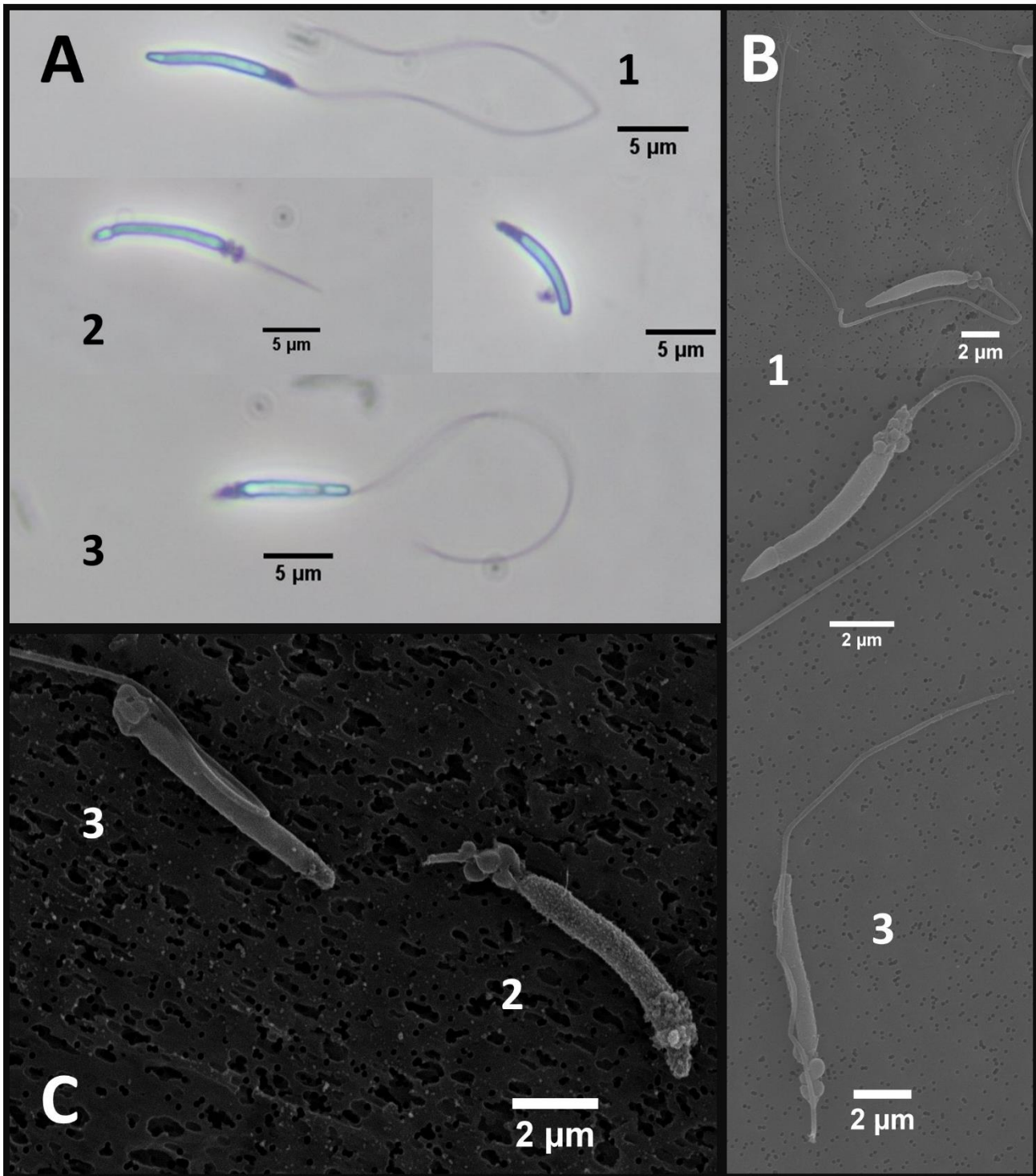


Figure 21: Tail defects of North African houbara bustards' sperm observed with contrast phase microscopy (oil immersion, x1000) (A), SEM (B, C) – 1: bended tail in the length, 2: broken tail, 3: coiled tail at the base of the midpiece.

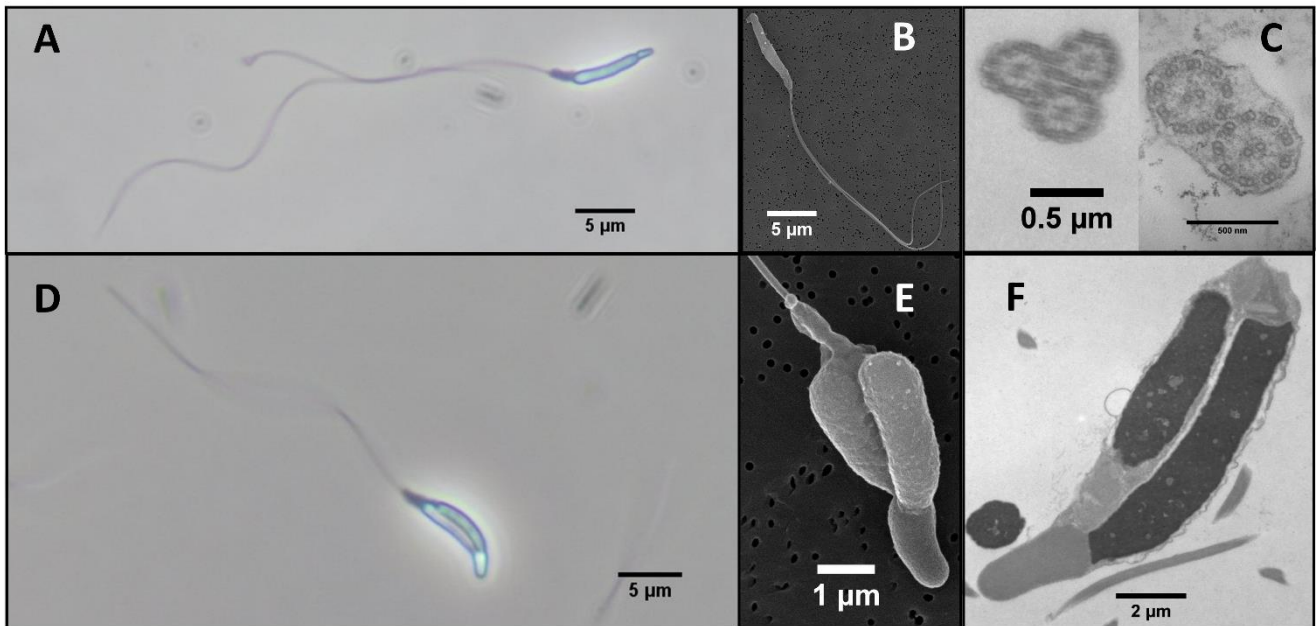


Figure 22: Teratogenic defects of North African houbara bustards' sperm: spermatozoa with double (or triple) tail observed with contrast phase microscopy (under oil immersion, x1000) (A), SEM (B) and TEM (C); spermatozoa with double head observed with contrast phase microscopy (under oil immersion, x1000) (D), SEM (E) and TEM (F).



Tables

Table I: Measurements (length in mm) of oviduct segments during breeding and non-breeding seasons (NBS)

	BS						NBS
	Vagina (N=12)	Uterus (N=12)	Isthmus (N=14)	Magnum (N=14)	Infundibulum (N=14)	Total length (N=12)	Total length (N=2)
Mean ± SD	21.41 ± 6.61	58.66 ± 11.80	65.76 ± 15.92	237.55 ± 50.37	37.08 ± 13.52	420.48 ± 70.52	76.31 ± 16.47
Min - Max	12.45 - 31.99	42.12 - 78.14	41.56 - 91.28	121.42 - 302.33	16.22 - 66.13	268.49 - 513.65	64.66 - 87.96

Table II: Measurements (thickness in μm) of each layer of the different oviduct segments during breeding (BS) and non-breeding seasons (NBS)

Layer thickness in μm		Oviduct segment									
		Infundibulum		Magnum		Isthmus		Uterus		Vagina	
		BS (N=40)	NBS (N=10)	BS (N=60)	NBS (N=20)	BS (N=60)	NBS (N=20)	BS (N=60)	NBS (N=20)	BS (N=30/20)*	NBS (N=20)
Mucosa	Mean ± SD	183 ± 59.3	65.9 ± 26	533 ± 189	104 ± 21.7	285 ± 83.1	92.5 ± 43.1	213 ± 71.7	75.3 ± 32.8	659 ± 398	190 ± 115
	Min - Max	83.8 - 320	34.9 - 102	196 - 1193	73.1 - 151	137 - 515	51.8 - 226	77.2 - 489	30.9 - 152	125 - 1368	58.5 - 551
Submucosa	Mean ± SD	66.3 ± 24.3	32.5 ± 12.8	76 ± 42.4	83.5 ± 27.7	48.2 ± 20	52.1 ± 21.4	56.4 ± 23.9	103 ± 40.4	170 ± 105	107 ± 30.3
	Min - Max	16.5 - 132	16.4 - 62.5	19.8 - 197	42.4 - 155	17.3 - 107	24 - 90.1	28.3 - 145	43.1 - 191	31.1 - 378	60.7 - 169
Muscularis	Mean ± SD	181 ± 51.6	67.9 ± 16.3	128 ± 52.6	175 ± 67.9	244 ± 79.1	125 ± 22.2	658 ± 251	542 ± 104	2228 ± 339	1145 ± 274
	Min - Max	97.3 - 288	41.6 - 96.7	66.5 - 326	55.5 - 293	145 - 549	89.7 - 168	342 - 1240	280 - 817	1637 - 3030	595 - 1498
Serosa	Mean ± SD	31.1 ± 20	13.8 ± 2.76	24.6 ± 8.37	11.9 ± 4.27	32.1 ± 15	11.3 ± 2.66	33.4 ± 15.1	32.6 ± 11.7	64 ± 23.2	49.1 ± 13.6
	Min - Max	12.1 - 92.6	8.16 - 18.6	11.4 - 43.6	7.01 - 23.1	12.6 - 77.5	5.08 - 15.5	15.1 - 99.9	17.7 - 59	39.2 - 133	31 - 75.2

Comparison between BS and NBS: Alternative hypothesis H_a BS > NBS, Mann-Whitney U test: U=1456, $p < 0.001$ (Mucosa thickness), U=10372, $p = 0.290$ (Muscularis thickness), U=9001, $p = 0.998$ (Submucosa thickness), U=6940, $p < 0.001$ (Serosa thickness). * During breeding season, the measurement of the vagina corresponds to N=30 for the mucosa and submucosa layers and N=20 for the muscularis and serosa layers.

Table III: Testis and epididymis measurements (mm) and testis colour grade during breeding (BS) and non-breeding seasons (NBS)

	BS (N=20 testis)		NBS (N=8 testis)	
	Testicular capsule thickness (μm) (n=200)	Seminiferous tubules diameter (μm) (n=200)	Testicular capsule thickness (μm) (n=80)	Seminiferous tubules diameter (μm) (n=80)
Mean ± SD	52.5 ± 17.9	128 ± 22.5	55.1 ± 26.1	97.9 ± 17.5
Min - Max	14.8 - 116	82.6 - 208	18.1 - 146	57 - 137

Comparison between BS and NBS: Alternative hypothesis H_a BS > NBS, Mann-Whitney U test $U=47.0$, $p=0.023$ (length testis), $U=9.00$, $p<0.001$ (width testis).

Comparison between left vs right side: Alternative hypothesis H_a Left > Right, Student's T test: $t=0.580$, $p=0.283$ (length testis), Mann-Whitney U test: $U=-135$, $p=0.913$ (width testis).

Table IV: Measurements (μm) of the testicular capsule thickness and seminiferous tubules (ST) diameter during breeding and non-breeding seasons (NBS)

		Testis				Epididymis				Testis Colour Grade (1-6)
		Length		Width		Length		Width		
		Right	Left	Right	Left	Right	Left	Right	Left	Right and left
BS	Mean \pm SD	15.91 \pm 2.95	16.65 \pm 3.22	7.24 \pm 1.03	6.76 \pm 1.08	24.65 \pm 4.46	24.98 \pm 6.19	4.58 \pm 1.35	4.28 \pm 0.96	1.84 \pm 1.17
	Min – Max	11.90-22.50	12.40-23.80	6.00-9.60	5.30-9.10	15.10-33.80	11.50-37.70	3.20-8.90	3.20-5.50	1-5.50
	N	N= 13	N= 14	N= 15	N= 16	N= 16	N= 15	N= 16	N= 16	N=21
NBS	Mean \pm SD	13.66 \pm 4.09	13.26 \pm 4.06	5.78 \pm 0.30	4.35 \pm 0.68	NA	NA	NA	NA	1.25 \pm 1
	Min – Max	10.57-19.63	10.44-17.91	5.44-6.14	3.60 -4.91					1-2
	N	N= 4	N= 3	N= 4	N= 3					N= 4

N=20 testis (BS) and N=8 testis (NBS), 10 ST measured per testis. Comparison between BS and NBS: Alternative hypothesis H_a BS > NBS, Mann-Whitney U test: $U=7825$, $p=0.388$ (testicular capsule thickness), $U=2305$, $p<0.001$ (ST diameter).

Table V: Houbara sperm morphometry: measurement (μm) of each sperm cell segment

- a. Evaluated with phase contrast microscopy, x400, N=440 from 22 males (20 spermatozoa per male)

Length in μm (N=440)	Acrosome	Head	Midpiece	Tail	Total length
Mean \pm SD	1.25 \pm 0.12	6.13 \pm 0.59	1.88 \pm 0.15	37.08 \pm 2.46	46.34 \pm 2.75
Min - Max	0.91 - 1.50	5.12 – 7.18	1.64 - 2.27	33.26 - 41.79	41.38 - 51.44

- b. Evaluated with scanning electron microscopy, N=40 spermatozoa from sperm mixes

Length in μm (N= 40)	Acrosome	Head	Midpiece	Tail	Total length
Mean \pm SD	1.51 \pm 0.50	6.69 \pm 2.14	2.49 \pm 0.64	32.87 \pm 6.58	43.56 \pm 7.19
Min - Max	0.79 - 2.70	3.76 - 11.65	1.66 - 3.95	17.62 - 45.98	31.09 - 59.55

Table VI: Percentage of normal and different abnormal sperm morphology categories in the North African Houbara Bustard – 100 sperm cells evaluated per sample (phase contrast microscopy), N=417 ejaculates from 166 male breeders.

%	Normal	Acrosome absence	Cytoplasmic droplet	Head swollen	Head abnormality	Midpiece defect	Tail defect	Teratogenic
Mean ± SD	68.5 ± 14.7	3.48 ± 4.12	2.08 ± 1.89	3.88 ± 9.03	5.35 ± 4.27	8.06 ± 7.19	4.57 ± 5.22	4.07 ± 4.49
Min - Max	3 - 92	0 - 59	0 - 9	0 - 80	0 - 29	0 - 57	0 - 42	0 - 37



CHAPTER 2: EXPLORING THE MECHANISMS UNDERLYING THE LAST MALE PRECEDENCE IN THE NORTH AFRICAN HOUBARA BUSTARD

Léna Meunier^{1,2}, Gabriele Sorci², Caroline Silva Vieira^{1,3}, Sadiya Sadiq Shiek¹, Yves Hingrat¹, Michel Saint Jalme¹, Janaina Torres Carreira^{1*}

¹ Reneco International Wildlife Consultants LLC, Abu Dhabi, United Arab Emirates

² Biogéosciences, UMR 6282 CNRS, Université de Bourgogne, Dijon, France

³ Federal University of Minas Gerais, UFMG, Belo Horizonte-MG, Brazil

*corresponding author, jtcarreira@reneco.org

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Summary statement

Utilizing nuclear and mitochondrial fluorescent stains, the study revealed that active sperm displacement during successive matings is likely the primary mechanism behind last male precedence effect in the North African Houbara Bustard.

Abstract

The last male precedence (LMP) effect refers to the competitive advantage of the last male in the mating sequence. Here, we conducted *in vitro* and *in vivo* experiments to explore the mechanisms underlying the LMP in a polyandrous bird, the North African Houbara Bustard (*Chlamydotis undulata*). We stained ejaculates with two nuclear fluorescent stains [Hoechst 33342 (BLUE), and NUCLEAR-ID[®] Red DNA (NRED)] and a mitochondrial stain [MitoTracker[™] Red FM (MRED)], and we compared the motility and velocity of stained and unstained sperm. Given that NRED impaired sperm motility and velocity, BLUE and MRED were retained for the *in vivo* experiments. First, we inseminated females with the sperm of a single male stained with BLUE and counted the number of sperm in the storage tubules at 24, 48 and 72 hours post-insemination. We found similar number of stored sperm suggesting that sperm loss does not occur over a 72 hours period. Second, we inseminated females with BLUE stained



sperm and, after 48 hours, females were inseminated again with MRED stained sperm. Twenty-four hours after the second insemination, we counted the number of stored sperm, and assessed their location within the tubules and their distribution across tubules. The majority of tubules (89 %) were filled with BLUE and MRED stained sperm. However, tubules contained more sperm from the second insemination and they were located closer to the bottom of the tubule. Therefore, active sperm displacement during successive mating is likely to be the principal mechanism underlying the LMP in this species.



Introduction

In many species, females have the ability to store sperm inside their reproductive tract, allowing fertilization of an egg even long after mating (Bakst *et al.* 1994; Holt & Fazeli 2016). In birds, sperm are stored inside specialised structures located inside mucosa folds of the utero vaginal junction (UVJ), called sperm storage tubules (SSTs) (Blesbois & Brillard 2007). The duration of sperm storage and the number of SSTs vary among species (Birkhead & MØLLER 1992). For instance, Ring doves (*Streptopelia risoria*) can store sperm up to 6 days (Zenone *et al.* 1979), Japanese quails up to 12 days (*Coturnix japonica*) (Reddish *et al.* 1996), while storage can extend up to 72 days in Turkeys (*Meleagris gallopavo*) (Lorenz 1950). Concerning the number of SSTs, it ranges from a few hundred in passerines (Briskie 1996a), 2000 to 3000 in the chicken (*Gallus domesticus*) (Brillard *et al.* 1998), and up to 20000 in the Turkey (Goodrich-Smith & Marquez 1978). In polyandrous species, females mate with multiple males and a post-copulatory sexual selection occurs as sperm from different males can be stored in the SSTs and compete for fertilization. When females mate with multiple males, it is commonly reported that the last male in the mating sequence has a competitive advantage, as it has a higher likelihood to fertilize the eggs (Birkhead 1998). This widespread phenomenon is known as the last male precedence (LMP) (Birkhead).

The mechanisms accounting for the competitive advantage of the last male have been less studied, although several hypotheses have been put forward (Birkhead & Biggins 1998). The first hypothesis assumes that stored sperm are progressively lost over time (at a constant or accelerating rate). Therefore, depending on the time interval between matings, and the rate at which sperm is lost, sperm from the last male are likely to outnumber the sperm from the previous males. The second hypothesis assumes that during successive matings, the newly ejaculated sperm will displace the previously stored sperm. Therefore, sperm from the last male merely replace previously stored sperm inside the SSTs. The third hypothesis assumes that sperm are stratified inside the SSTs depending on the mating order. According to this hypothesis, sperm from the first male are stored deep in the SSTs, while sperm of the last male are stored closer to the SST opening and therefore will be released first for fertilization. A final hypothesis assumes that SSTs closer to the vagina are filled with sperm from the first mating male, whereas sperm from the last male are stored in SSTs closer to the uterus (because those close to the vagina are already filled). When this spatial segregation occurs, sperm stored closer to the fertilization site might have a competitive advantage compared to those stored more distantly (Briskie 1996b).

Sperm loss has been considered as the predominant mechanism explaining LMP in birds (Birkhead & Biggins 1998). However, only a handful of studies have used direct methods to



differentiate sperm of different males inside the storage structures, which limits our understanding of LMP in birds. Van Krey *et al.* (1981) performed inseminations with a combination of radiolabelled (3H-thymidine) and unlabelled sperm in the fowl and found no evidence of sperm displacement. King *et al.* (2002) performed tandem inseminations with sperm labelled with Hoechst 33342 and unstained sperm in the fowl and the turkey and showed that sperm from subsequent inseminations segregated in different SSTs. Finally, more recently, Matsuzaki *et al.* (2023) used a double fluorescent sperm labelling to investigate LMP in the fowl. They found that after two consecutive inseminations with a three-day interval, more than 70% of sperm from the first insemination was replaced by sperm of the second one. They also reported evidence showing that the number of stored sperm decreased over the first 5 days after insemination. Based on this sparse experimental evidence, it is difficult to have a clear picture of the relative importance of the mechanisms underlying LMP in birds. Work done on invertebrates provides additional information on the proximal factors underlying LMP. For instance, using transgenic lines of *Drosophila melanogaster* expressing fluorescent proteins on sperm, Manier *et al.* (2010) showed that sperm of the first male are physically displaced by sperm of the second one, in agreement with the displacement hypothesis.

As mentioned above, testing the different hypotheses underlying the LMP is challenging because we need to differentiate sperm from different males within the SSTs. Fluorescent staining allows to label sperm and visualize them inside SSTs. However, fluorochromes must meet a series of criteria to be suitable for *in vivo* studies. In particular, the staining must label all sperm in the ejaculate, not impair sperm motility or viability and the fluorescent signal should last long enough to allow sperm visualization in the storage structures (Henning *et al.* 2019).

Here, we aimed at investigating the mechanisms underlying the LMP in a wild bird species with a polyandrous mating system, the North African Houbara Bustard (*Chlamydotis undulata*). The natural populations of this species have declined over the last decades due to habitat destruction and overhunting (2023). An *ex-situ* conservation breeding program was established in Morocco to restore declining populations through translocation of captive bred houbara (Lacroix 2003). During the breeding season, lasting from January to June in Morocco, male houbara bustards display in specific sites [exploded leks (Hingrat & Saint Jalme 2005)], and females choose their mate (Hingrat *et al.* 2007). Females can copulate with several males as multiple paternities have been reported in the wild (Lesobre *et al.* 2010). Females lay one clutch of 2 to 4 eggs with a delay of 1 to 3 days between eggs. If a clutch is lost a replacement clutch is laid (Saint Jalme *et al.* 1994). The reproduction in captivity is artificial with the insemination of females using sperm collected from males housed separately. Using data collected over the last 20 years, Sorci *et al.* (2023) showed that when females are inseminated



with different males, the last male in the insemination sequence has more than sixty percent likelihood to fertilize the eggs showing that the LMP is operating in this species.

To unravel the mechanisms driving the LMP in the North African Houbara Bustard and to investigate whether sperm from different males can be found on the egg perivitelline membrane, we first conducted *in vitro* experiments to select fluorescent dyes suitable for inseminations with stained sperm. Then, we conducted non-competitive (involving a single male) and competitive (involving two males) inseminations, using stained sperm. Specifically, we formulated the following predictions. If passive sperm loss leads to the LMP, the number of sperm stored in the SSTs should decrease with time post-insemination. If sperm displacement occurs, sperm stored in the SSTs from the last insemination should outnumber sperm from the first insemination. If sperm stratify inside SSTs, sperm from the first insemination should be at the bottom of SSTs whereas sperm from the second insemination should be closer to the tubule opening. If sperm segregation occurs, sperm from different inseminations should be separately stored inside different SSTs.

Material and Methods

Experimental birds

All birds used in this study were part of a conservation breeding program at the Emirates Center for Wildlife Propagation (ECWP), in eastern Morocco, aiming at reinforcing natural populations of the North African Houbara Bustard (Lacroix 2003). All birds were kept in 4 square meters individual cages and received commercial specially formulated dry food pellets, mealworms, and crickets as food. All males used for the *in vitro* sperm staining experiments and the *in vivo* experiments were part of the breeding flock. The females used for the *in vivo* experiments were born in captivity and were of generation F3 or more. They were deemed to be culled for the purposes of the management of the captive flock; no bird that could have either be released in the wild or kept as future breeder was euthanized. A total of 14 sexually mature females [mean age (yrs) \pm sd = 5.6 \pm 0.9] were culled for this study.

Semen collection

Ejaculates were collected with a dummy female presented to the male, following a standard procedure. Briefly, when the male mounts the dummy and starts copulating, a glass petri dish is used to collect the ejaculate by pressing it gently on the male cloaca. The ejaculate was immediately transferred to a 2 mL cryotube, and diluted (1:1) in Lake 7.1 (Lake & Ravie 1984). Samples were maintained at room temperature (20° C, < 1h) until processed for fluorescent staining.



In vitro experiments

To distinguish sperm of different males, we first evaluated three fluorescent stains, two nuclear [Hoechst 33342 (Invitrogen, USA) and NUCLEAR-ID® Red DNA (Enzo, USA)] and one mitochondrial stain [MitoTracker™ Red FM (Invitrogen, USA)], hereafter called BLUE, NRED and MRED, respectively. Prior to using them for *in vivo* inseminations, we tested the effect of the staining on sperm motility and velocity (using unstained sperm as control) and the persistence of the fluorescent signal over time.

Effect of BLUE vs. NRED staining on sperm motility and velocity

Ejaculates from 20 males (mean concentration \pm sd of the diluted ejaculates = $552 \pm 110 \times 10^6$ sperm per mL) were used. Each sample was split into three aliquots, one was stained with BLUE (350 nm excitation/461 nm emission), one with NRED (566 nm excitation/650 nm emission) and the third aliquot was used as an unstained control (Fig. 1A). Both fluorochromes had a final concentration of 20 μ M. After 20 minutes of incubation in the dark, at room temperature (20° C), samples were washed (centrifuged 5 minutes at 1000 g), resuspended in PBS/BSA 1.5% and sperm motility and velocity was assessed. Then, each aliquot was divided in two and kept either at 4° C or 40° C. The persistence of the fluorescence signal was assessed under the microscope 24, 48 and 72 hours post-incubation. Motility and velocity were also assessed 24, 48 and 72 hours post-incubation but only on samples kept at 4° C. Indeed, although 40° C correspond to the mean body temperature, at this temperature sperm progressively become immotile (sperm are also immotile inside the SSTs). Avian sperm storage at low temperature is commonly used to preserve motility and limit bacterial growth (Blesbois & Brillard 2007; Wishart 2009).

Sperm motility and velocity were assessed with the Sperm Class Analyzer software (SCA, Microptics, Barcelona, Spain). One aliquot of each treatment (BLUE, NRED, MRED and unstained controls at each time interval) was diluted (1:10 in PBS/BSA 1.5%) and four microliters were placed on a 20 μ m depth disposable chamber (Proiser, Arquimea, Spain) under a negative phase contrast microscope (Nikon E200-LED, Japan, objective x10). A maximum of 30 fields were analysed as to reach 500 sperm (see Supplementary Table 1 for details on SCA settings). We recorded the percentage of motile sperm and their curvilinear velocity [VCL (μ m/s)].

Effect of MRED staining on sperm motility and velocity

Ejaculates from 17 males (mean concentration \pm sd of the diluted ejaculates = $278 \pm 105 \times 10^6$ sperm per mL) were used. Each sample was split into two aliquots, one was stained with MRED (581 nm excitation /644 nm emission) at a final concentration of 1 μ M and the other aliquot was used as an



unstained control (Fig. 1B). After 20 minutes of incubation in the dark at room temperature (20° C), samples were washed (centrifuged 5 minutes at 1000 g), resuspended in PBS/BSA 1.5% and sperm motility and velocity were assessed as described above. Motility, velocity and the persistence of the fluorescence signal were assessed again 24, 48 and 72 hours after.

In vivo experiments

Sperm loss

To investigate sperm loss over time, 9 females were inseminated with 40×10^6 sperm stained with BLUE. Females were euthanized at 24, 48, and 72 hours post-insemination to count the number of stained sperm stored in the SSTs (Fig. 1C). Birds were euthanized by veterinarians of the ECWP by an intravenous injection of one mL of T61 (MSD, 200 mg Embutramide, 50 mg Mebezonium Iodide and 5 mg Tetracaine Hydrochloride) (Baker-Cook *et al.* 2021). Immediately after euthanasia, the coelom was opened, the oviduct harvested, and the utero vaginal junction (UVJ) was removed, kept in PBS 1X and transferred to the laboratory. The UVJs were dissected under a stereomicroscope (MZ9.5, Leica, Switzerland) within one hour from collection. Mucosa folds containing SSTs were identified with Leica Application Suite software EZ software (version 3.2.1, Leica, Switzerland). Ten mucosa folds per female were placed on Poly-L-Lysine coated slides with one drop of mounting media (1:1 Glycerol and PBS 1X) and a cover slip. Slides were observed under a fluorescent microscope (Nikon E200-LED, Japan; fluorescent light: CoolLED pE-300-W-EPI-Fluorescence LED illumination system and module: Nikon EPI-Fluorescence module CI-FL) equipped with a camera. For each female, a maximum of 10 SSTs (containing at least one sperm) per UVJ mucosa fold were observed and photographed using a blue filter (Chroma AT-UV/DAPI Longpass filter block) and a red filter (Texas red filter). The number of stained sperm in each SST was then counted on the photographs.

Sperm displacement, segregation, and stratification

To investigate sperm displacement, stratification, and segregation, two females were first inseminated with 40×10^6 BLUE stained sperm, and after 48 hours, with 40×10^6 MRED stained sperm, collected from a different male (Fig. 1D). Twenty-four hours after the second insemination, females were euthanized, and the oviduct collected and processed as described above. We counted the number of differently stained sperm. To control for a possible negative effect of the staining on sperm attributes, a control group was formed by three females that were simultaneously inseminated with a mix of 20×10^6 BLUE and 20×10^6 MRED stained sperm collected from two males. These females were also euthanized 24 hours post-insemination and processed as described above. To assess the position of stained sperm within SSTs, a total of ten SSTs were selected for each female and the distance (μm) of each sperm (head and midpiece junction used as measuring point) from a fixed point of the dead



end of the SST was computed using Fiji software (Schindelin *et al.* 2012, December 2020 version). The distance was measured using segmented lines to adjust to the shape of the SSTs.

Stained sperm on the perivitelline membrane

One female was inseminated with a mix of 20×10^6 BLUE and 20×10^6 MRED stained sperm collected from two males and was allowed to lay. One egg was collected the day of laying and processed as to visualize the presence of stained sperm. Another egg was found inside the uterus of one of the control females inseminated with a mix of stained sperm, and used to visualize the presence of stained sperm.

Eggs were assessed for fertility and the presence of sperm on the perivitelline layer. Briefly, the yolk was transferred inside a glass container filled with PBS 1X. The perivitelline membrane surrounding the embryonic disk was cut with fine scissors and after removing yolk excess, was placed on Poly-L-Lysine coated slides with one drop of mounting media (1:1 Glycerol and PBS 1X) and a cover slip. The presence of stained sperm was checked under fluorescent microscope (Nikon E200-LED, Japan; fluorescent light: CoolLED pE-300-W-EPI-Fluorescence LED illumination system and module: Nikon EPI-Fluorescence module CI-FL) using a blue (Chroma AT-UV/DAPI Longpass filter block) and a red (Texas red filter) filter. The presence of embryonic cells was later checked by adding a drop of DAPI mounting media (Fluoroshield, Abcam, USA) on the slide.

Statistical analyses

Motility and velocity (response variables) of stained (BLUE, NRED, and MRED) and unstained sperm were analysed using General Linear Mixed Models (GLMM) with a normal distribution of errors, where the identity of the ejaculate donor was declared as a random effect and the staining group as a fixed effect. Least-square means were compared between groups and p-values adjusted for multiple comparisons (Bonferroni correction). Changes in motility and velocity over time for stained and unstained sperm were modelled using the same GLMM with the addition of time and group by time interaction as fixed effects.

The number of sperm stored in the tubules (response variable) was modelled using a Generalized Linear Mixed Model with a Poisson distribution of errors. The model included female identity, the identity of the mucosa fold (1 to 10) nested within female, and the identity of the tubule (1 to 10) nested within fold and female as random effects. Time post-insemination (24, 48, and 72 hours), or insemination order, and date of insemination were included as fixed effects.



The hypothesis that sperm from the two inseminations might segregate in different tubules was investigated comparing the observed distribution of tubules containing BLUE sperm alone, MRED sperm alone, or both types of sperm with a random distribution (1/3 for each category), using a χ^2 test. The distance of BLUE and MRED sperm from the bottom of the tubule (response variable) was modelled using a General Linear Mixed Model with a normal distribution of errors. The model included female identity and the identity of the tubule (1 to 10) nested within female as random effects. The staining group was included as a fixed effect. Statistical analyses were done in SAS (9.4).

Ethical statement

The breeding program runs under the approval of the Moroccan Ministry of Agriculture and a mandate independent veterinarian (mandate number: 534-98), providing control of the well-being of the birds. Onsite veterinary facilities ensure the best possible care for sick or injured birds by a team of expert veterinarians, and standards from sanitary authorities are regularly controlled. Experiments are conducted in an ethical manner, according to the Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes (<http://data.europa.eu/eli/dir/2010/63/oj>), and each breeding protocol is developed while ensuring appropriate and ethical treatment of birds. To minimize stress during capture, birds are fitted with hoods during manipulation. Facilities are designed to ensure the well-being of the birds and prevent injuries.

Results

In vitro experiments

Effect of BLUE vs. NRED staining on sperm motility and velocity

After 20 minutes of incubation at room temperature, there were no statistically significant differences in total motility (Fig. 2A) and VCL (Fig. 2B) between sperm stained with BLUE and unstained sperm ($t = 1.60$, adjusted $p = 0.35$, and $t = 1.00$, adjusted $p = 0.97$, respectively). However, sperm stained with NRED had statistically significant lower motility and VCL compared to unstained sperm ($t = -3.05$, adjusted $p = 0.01$, and $t = -5.09$, adjusted $p < 0.01$, respectively).

Over time motility decreased at a statistically significant faster rate for sperm stained with NRED than for sperm stained with BLUE or unstained sperm (time by group interaction: $F_{4,152} = 2.92$, $p = 0.02$; Fig. 2C). The rate of VCL decline over time was not statistically different between the three staining groups (time by group interaction: $F_{4,152} = 1.92$, $p = 0.11$) (Fig. 2D).



The fluorescent signal persisted for up to 72 hours at both temperatures (4° and 40° C) for the two fluorochromes (Supplementary Fig. 1).

Effect of MRED staining on sperm motility and velocity

After 20 minutes of incubation at room temperature, there was no statistically significant difference in total motility (Fig. 3A) and VCL (Fig. 3B) between samples stained with MRED and unstained samples ($t = -0.59$, $p = 0.56$; $t = -0.03$, $p = 0.98$). Total motility (Fig. 3C) and VCL (Fig. 3D) decreased with time at a statistically significant faster rate for sperm stained with MRED than for unstained sperm (time by group interaction: motility, $F_{8,80} = 3.43$, $p = 0.03$; VCL, $F_{8,80} = 4.82$, $p = 0.01$).

The fluorescent signal persisted for up to 72 hours at both temperatures (4° and 40° C).

In vivo experiments

Sperm loss

The number of stained sperm counted in the SSTs did not significantly change over time, up to 72 hours post-insemination ($F_{2,4.72} = 1.77$, $p = 0.26$; Fig. 4).

Sperm displacement, segregation and stratification

Sperm stained with BLUE appeared with a bright blue nucleus and sperm stained with MRED had a red midpiece. Sperm with a red midpiece also showed a light blue nuclear staining due to a BLUE stain transfer. However, this did not prevent sperm assignment. Both BLUE and MRED stained sperm were found in 85% of the SSTs of females inseminated with sperm mixes (Fig. 5). Significantly more sperm stained with BLUE were found in the SSTs after insemination with sperm mixes (least squares means (95% CI) per SST = BLUE, 8.19 (2.62 - 25.64); MRED, 4.33 (1.39 - 13.47), $F_{1,597} = 502.46$, $p < 0.01$).

Females inseminated at 48-hour interval stored significantly more sperm from the second insemination (stained with MRED) compared to sperm from the first insemination (stained with BLUE) ($F_{1,255} = 882.80$, $p < 0.01$; Fig. 6A and Fig. 7).

We did not find evidence suggesting that sperm from first and second inseminations might segregate in different tubules as 89% of the tubules contained both MRED and BLUE stained sperm. Under the hypothesis of spatial segregation we would expect a majority of tubules containing either BLUE or MRED stained sperm. Contrary to this prediction, we found a statistically significant excess of tubules containing sperm stained with the two fluorochromes (percent of tubules with only MRED sperm = 3.1 %; percent of tubules with only BLUE sperm = 7.8 %; percent of tubules with both sperm = 89.1 % $\chi^2_2 = 181.16$, $p < 0.01$).



According to the stratification hypothesis, sperm should be chronologically stored in the tubule from the bottom (first insemination) to the opening (last insemination). To explore this hypothesis, we measured the distance of each sperm from the bottom of the tubule. The stratification hypothesis predicts that this distance should be smaller for first inseminated sperm. Contrary to this prediction, we found that sperm from the second insemination were significantly closer to the bottom of the tubule compared to sperm from the first insemination ($F_{1,1197} = 35.13$, $p < 0.01$, Fig. 6B). In mixed inseminations, the distance from the bottom of the tubule did not significantly differ between sperm from first and second inseminations ($F_{1,964} = 0.22$, $p = 0.64$).

Stained sperm on the perivitelline membrane

The egg laid by the female inseminated with a mix of BLUE and MRED stained sperm had been fertilized since embryonic cells were observed upon adding DAPI on the slide. We also found several sperm with a light blue nucleus and a red midpiece on the egg membrane, corresponding to the MRED stained sperm. An additional egg was found inside the uterus of one female inseminated with a mix of BLUE and MRED stained sperm. Both MRED and BLUE stained sperm were found on the egg membrane (Supplementary Fig. 2). As for the previous egg, embryonic cells were also observed in this egg, showing that it had been successfully fertilized.

Discussion

Although the LMP has been reported in a diversity of organisms, the mechanisms underlying it have been difficult to elucidate. Several hypotheses have been put forward, including the passive loss of sperm stored in the SSTs, or the active displacement of stored sperm during successive matings. However, testing these hypotheses has proved challenging because of the technical difficulties to visualize competing sperm within the female reproductive tract. After conducting *in vitro* experiments to assess the suitability of different fluorescent staining, we used artificial inseminations with fluorescent stained sperm in the North African Houbara Bustard and demonstrated that the last male precedence in this species is likely due to the displacement of sperm stored in the SSTs.

To effectively stain sperm, we tested two nuclear fluorescent dyes and one mitochondrial staining. Total motility and VCL of sperm stained with BLUE were not different from unstained sperm over time. Moreover, BLUE produced a fluorescent signal that persisted up to 72 hours. Therefore, this fluorochrome was considered as a good candidate for *in vivo* use. This staining has also been used in the past in studies conducted on the chicken (McDaniel *et al.* 1997; King *et al.* 2002; Matsuzaki *et al.* 2023), the turkey (Bakst 1994) and the quail (Matsuzaki *et al.* 2021) and no sperm motility impairment



has been reported. It is also commonly used to stain mammalian sperm without impacting motility and it is even used in assisted reproduction techniques for sperm sex-sorting (Garner *et al.* 2013). The second nuclear staining that we tested, NRED, did impair motility and VCL and therefore was not considered suitable for *in vivo* use. In a recent paper, Matsuzaki *et al.* (2023) used two stains, a nuclear one (BLUE) and a cytosolic one (pHrodo-Red AM) to conduct artificial inseminations. However, pHrodo-Red AM emits a red fluorescence that depends on the pH of the cytosol, the stronger the acidity the higher the intensity of the signal. Since we do not have data on the pH of the oviduct in the Houbara Bustard, we preferred using dyes emitting fluorescence independently of pH conditions. Thus, we tested a mitochondrial staining (MRED) and found that motility and VCL were similar, 20 minutes after staining, for stained and unstained sperm. However, both motility and velocity decreased at a faster rate (from 24 to 72 hours post-insemination) compared to unstained sperm. Once sperm are stored inside the SSTs they enter into a resting phase and lose motility until fertilization of the oocyte (Matsuzaki & Sasanami 2022). Therefore, despite the faster rate of decrease in motility and VCL of MRED stained sperm, we chose the mitochondrial staining for *in vivo* use, based on the rationale that 24 hours post-staining sperm will be already stored in the SSTs (where they are no longer motile). The validity of the choice of the two fluorochromes for the *in vivo* experiments was confirmed by the observation of both MRED and BLUE stained sperm, not only inside the SSTs, but also on the perivitelline membrane. Up to now, only three fluorescent molecules, BLUE (King *et al.* 2002; Matsuzaki *et al.* 2021; Matsuzaki *et al.* 2023), pHrodo-Red AM (Matsuzaki *et al.* 2021; Matsuzaki *et al.* 2023) and MRED (present study) have been successfully used to study sperm storage in a competitive insemination context.

The results of the *in vivo* experiment suggest that there is no sperm loss over time up to 72 hours post-insemination. Therefore, for successive matings occurring within a period of two-three days, passive sperm loss is unlikely to account for the LMP effect. Passive sperm loss certainly operates on a longer time scale given that, although females can lay fertile eggs up to 47 days post-insemination, fertility decreases over time after insemination (Saint Jalme *et al.* 1994). Previous work has suggested that passive sperm loss might be the main mechanism underlying LMP in birds (Birkhead & Biggins 1998). To reach this conclusion, Birkhead and Biggins (1998) built a series of models that included passive sperm loss alone and passive sperm loss plus sperm stratification or displacement and used them to predict the expected paternity share under the different scenarios modelled. They then compared these predicted paternity shares with the observed ones derived from four published studies on poultry and turkey. The model that included the passive sperm loss alone provided the best fit to the observed paternity share (Birkhead and Biggins 1998). However, it should be noted that the model that included passive sperm loss plus displacement provided relatively similar predictions to the



model with passive sperm loss alone and it might be very difficult to tease apart the two models using datasets that have not been collected with the specific aim of testing them. Therefore, a direct assessment of the rate of sperm loss in the SSTs is needed to conclude on the importance of sperm loss compared to the other mechanisms.

When inseminated with a mix of sperm stained with the two fluorochromes, we expected to retrieve the same number of sperm in the SSTs. Contrary to this prediction, we found more sperm stained with BLUE compared to sperm stained with MRED. This difference is probably due to the effect of MRED on sperm motility, as reported *in vitro*. However, when sequentially inseminated with BLUE stained and MRED stained sperm, we consistently found more MRED stained sperm (those corresponding to the last insemination). These results, therefore, suggest that sperm transferred during successive matings may replace those previously stored in the SSTs. Therefore, active sperm replacement might be at play as a mechanism underlying LMP in the North African Houbara Bustard. A recent study in the chicken showed that more than 70% of sperm of a first insemination were replaced by sperm of a second insemination occurring 3 days after (Matsuzaki *et al.* 2023).

We did not find evidence supporting the spatial segregation hypothesis since the majority of tubules contained sperm from both the first and the last insemination. This result somehow contradicts previous work on the fowl and the turkey (King *et al.* 2002) which reported that only 4% and 12% of the SSTs were filled with sperm from different males. We did not find evidence for the stratification hypothesis neither. The stratification hypothesis posits that sperm pile up within the tubule according to the mating order with first transferred sperm being stored at the bottom of the tubule and last transferred sperm being stored close to the tubule opening. Contrary to this prediction, we found that sperm transferred during the second insemination were significantly closer to the bottom of the tubule compared to sperm transferred during the first insemination. Therefore, this finding does not support the stratification hypothesis, and rather corroborates the displacement hypothesis, as it suggests that, when entering SSTs, sperm of the last male might displace sperm of the first male previously stored, pushing them towards the tubule opening from where they might leak out.

We also report evidence showing that sperm stained with both fluorochromes were potentially able to fertilize the eggs. Several sperm (with the two dyes) were found on the perivitelline membrane and eggs were fertile, as embryonic cells were observed. Polyspermy refers to the entrance of multiple sperm inside the egg, however, only one sperm can fuse with the oocyte nucleus to form a zygote. Polyspermy represents a crucial physiological process in avian reproduction, essential for successful fertilization (Hemmings & Birkhead 2015). The presence of multiple sperm inside the perivitelline



membrane of the egg has been reported in several studies (Wishart 1987; Brillard & Bakst 1990; Brillard & Antoine 1990; Hemmings & Birkhead 2017). In the turkey, the number of sperm found on the perivitelline membrane appears to be positively correlated with the amount stored inside the SSTs (Brillard & Bakst 1990). In the Zebra finch (*Taeniopygia guttata*), sperm of two males, with different phenotypes, have been found on the perivitelline membrane, the higher the number of sperm of a given phenotype stored in the SSTs, the higher the number found on the PVM (Hemmings & Birkhead 2017). Here, the number of sperm on the PVM was not quantified, but it would be interesting to investigate whether the number of sperm reaching the egg differs when inseminations occur sequentially. This would provide strong evidence that the competitive advantage of the last male in the mating sequence is due to a larger number of sperm reaching the egg.

Conclusions

We provide evidence supporting the active displacement hypothesis as a possible mechanism underlying the competitive advantage experienced by houbara males who mate last in the mating sequence. We did not find evidence supporting the stratification or the segregation hypotheses. Passive sperm loss certainly occurs within the SSTs, as fertilization success declines as long as the delay between insemination and laying increases, with no fertile eggs being laid 47 days post-insemination. This might result from different processes such as the sperm leaking from the tubules or the death and removal of sperm by macrophages. However, we did not find evidence that sperm loss occurs over a short period of time, as the number of stained sperm retrieved at 24, 48 and 72 hours post-insemination did not differ significantly. Therefore, for matings occurring within a period of two-three days, passive sperm loss is unlikely to account for the LMP effect. We also reported evidence showing that sperm from different males do reach the perivitelline membrane. While polyspermy is a physiological process in birds, the presence of sperm of different males paves the way for competition occurring not only to reach the egg, but also within the egg.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.



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Author contribution statement

LM, YH, MSJ, GS, and JTC conceived the study; LM, CSV, and JTC collected the data, GS and LM analysed the data and prepared the tables and figures; LM, GS and JTC wrote the first draft of the manuscript; all authors edited, revised, and approved the final version.

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Figures

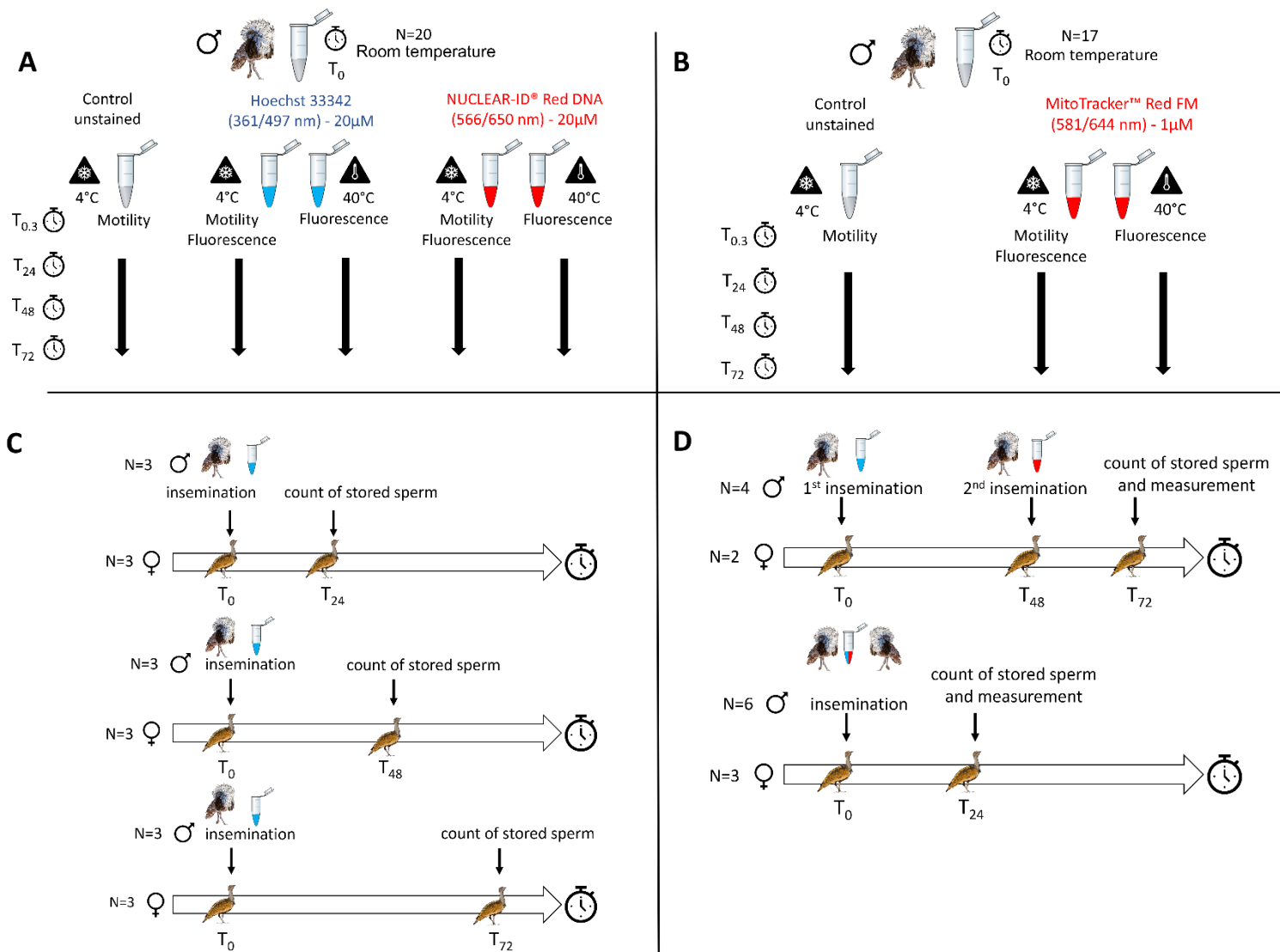


Figure 1: Experimental designs used for the *in vitro* and *in vivo* experiments, using captive male and female Houbara Bustards (*Chlamydotis undulata*). (A) Ejaculates (n = 20) were split into three aliquots and stained with Hoechst 33342 (BLUE), NUCLEAR-ID[®] Red DNA (NRED), or



left unstained. Sperm motility and velocity were measured at different time points post-staining at 4° C. The persistence of the fluorescent signal was also assessed at 40 ° C. (B) Ejaculates (n = 17) were split into two aliquots and stained with MitoTracker™ Red FM (MRED) or left unstained. Sperm motility and velocity were measured at different time points post-staining at 4° C. The persistence of the fluorescent signal was also assessed at 40 ° C. (C) Nine females were inseminated with BLUE stained sperm and the number of stained sperm stored in the sperm storage tubules counted at 24, 48, and 72 hours post-insemination. (D) Two females were inseminated with BLUE stained sperm and after 48 hours were inseminated again with MRED stained sperm. Twenty-four hours after the second insemination, the number of stained sperm stored in the sperm storage tubules was counted. A control group was constituted of three females that were simultaneously inseminated with a mix of BLUE and MRDED stained sperm. Twenty-four hours after the insemination, the number of stained sperm stored in the sperm storage tubules was counted.

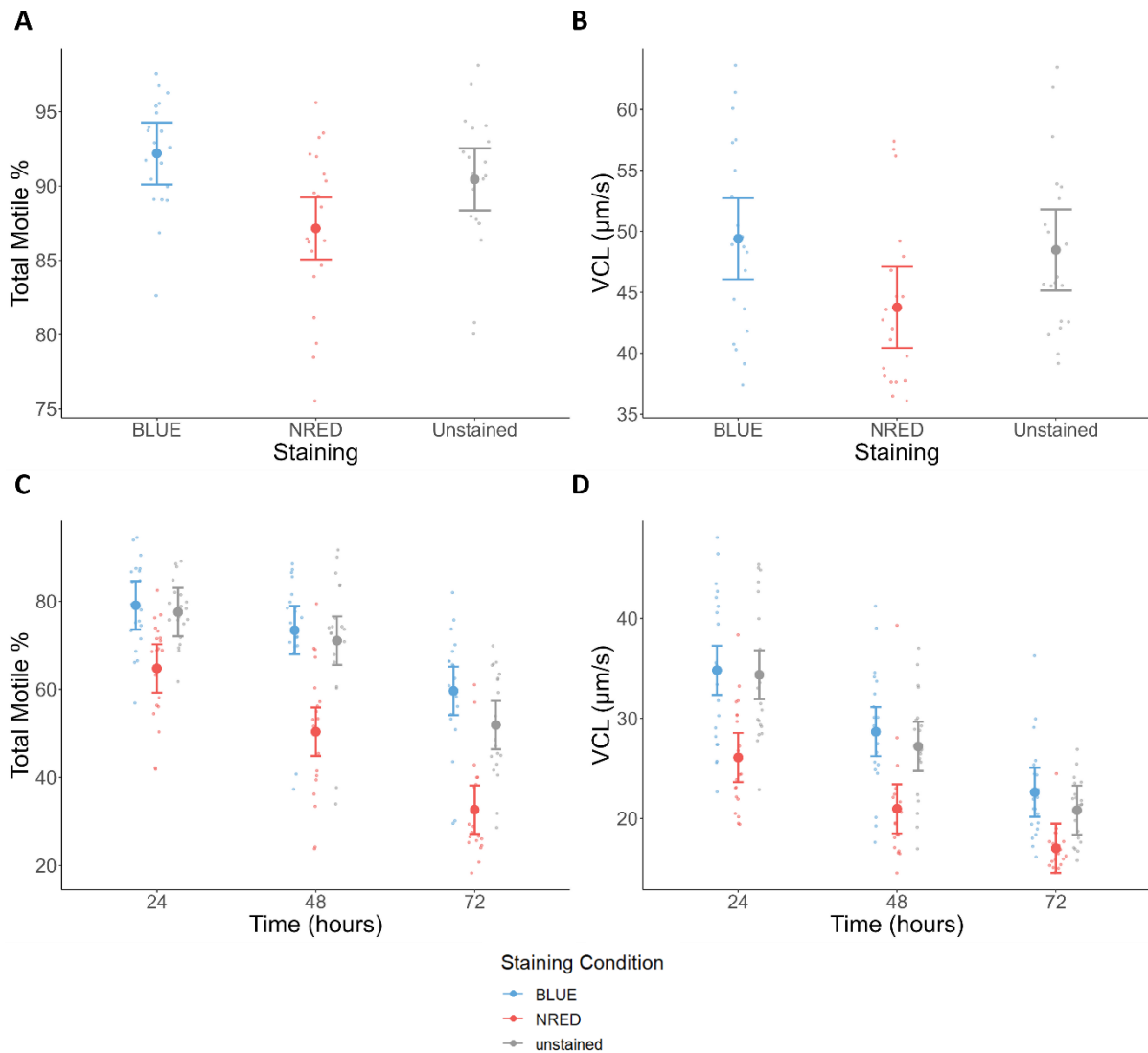


Figure 2: Total motility percentage (A) and curvilinear velocity (VCL in $\mu\text{m/s}$) (B) after 20 minutes of incubation at room temperature in the dark of Houbara Bustard (*Chlamydotis undulata*) sperm stained with 20 μM of BLUE, 20 μM of NRED or unstained. Total motility percentage (C) and curvilinear velocity (VCL in $\mu\text{m/s}$) (D) after 24, 48 and 72 hours of incubation at 4°C in the dark sperm stained with 20 μM of BLUE, 20 μM of NRED or unstained. Values are expressed as raw data (smaller dots), least-square means (larger dots) and lower and upper 95% confidence intervals (lower and upper error bars) for each group (n=20).

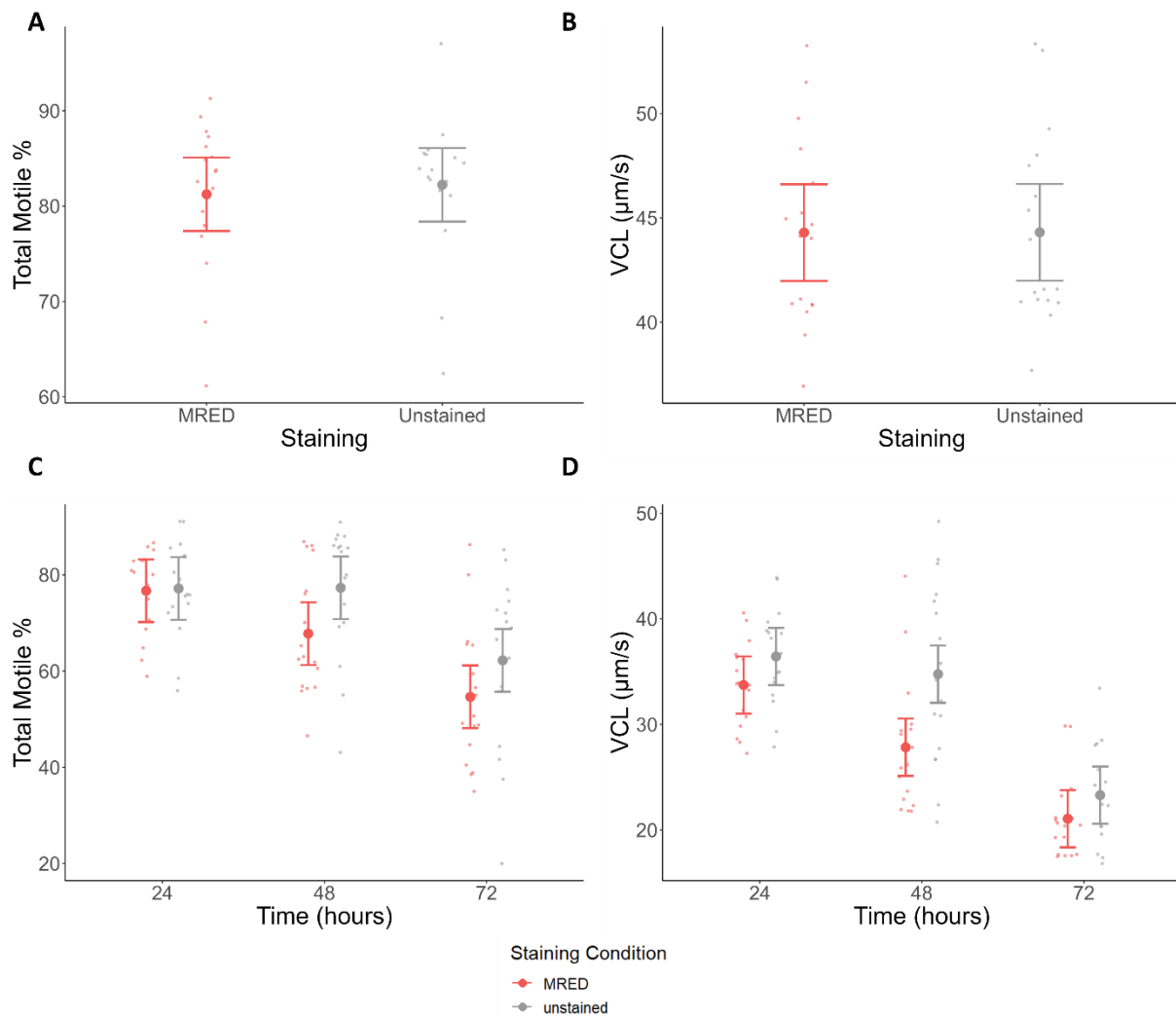


Figure 3: Total motility percentage (A) and curvilinear velocity (VCL in $\mu\text{m/s}$) (B) after 20 minutes of incubation at room temperature in the dark of Houbara Bustard (*Chlamydotis undulata*) sperm stained with $1 \mu\text{M}$ of MRED or unstained. Total motility percentage (C) and curvilinear velocity (VCL in $\mu\text{m/s}$) (D) after 24, 48 and 72 hours of incubation at 4°C in the dark of sperm stained with $1 \mu\text{M}$ of MRED or unstained. Values are expressed as raw data (smaller dots), least-square means (larger dots) and lower and upper 95% confidence intervals (lower and upper error bars) for each group (n=17).

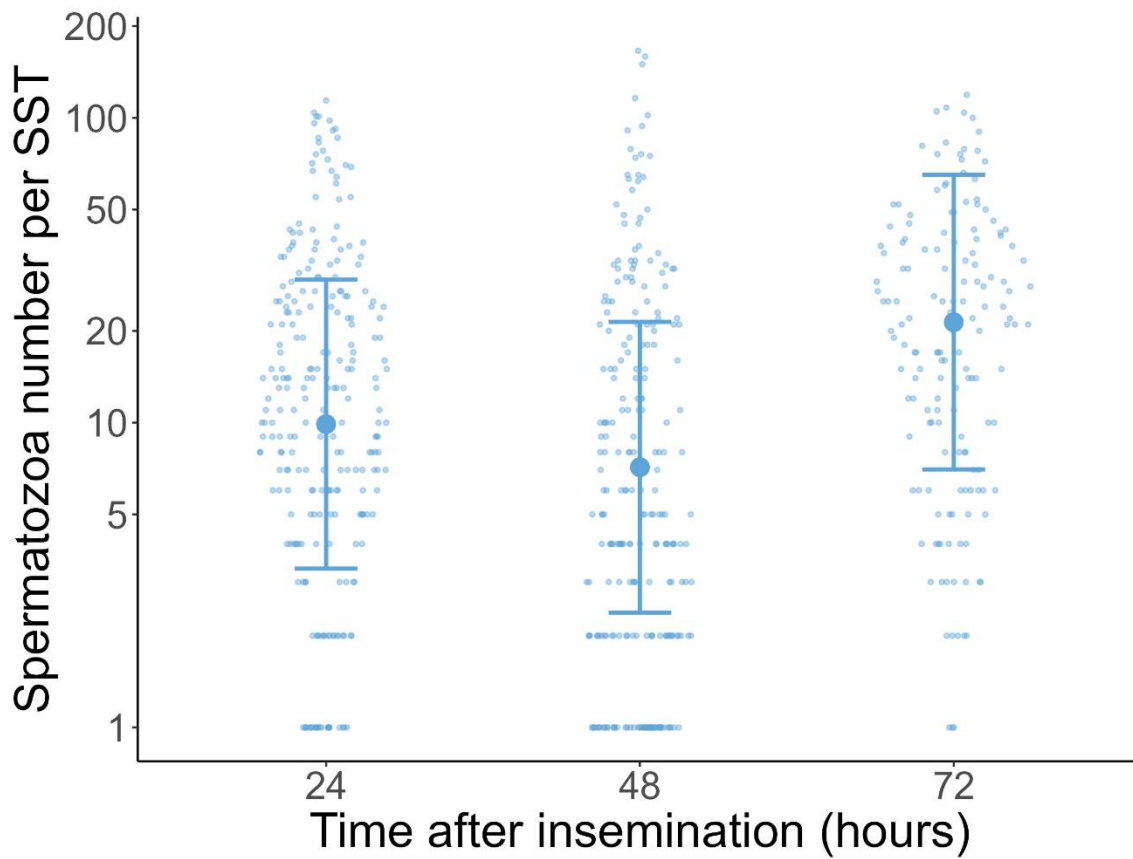


Figure 4: Number of Houbara Bustard (*Chlamydotis undulata*) sperm counted per SST at 24, 48 and 72 hours post-insemination. Values are plotted on a log₁₀ scale y axis and expressed as raw data (smaller dots), least-square means (larger dots) and lower and upper 95% confidence intervals (lower and upper error bars) for each time group (N=3 females and 253 SSTs at 24 h, 3 females and 248 SSTs at 48 h, 3 females and 156 SSTs at 72 h).

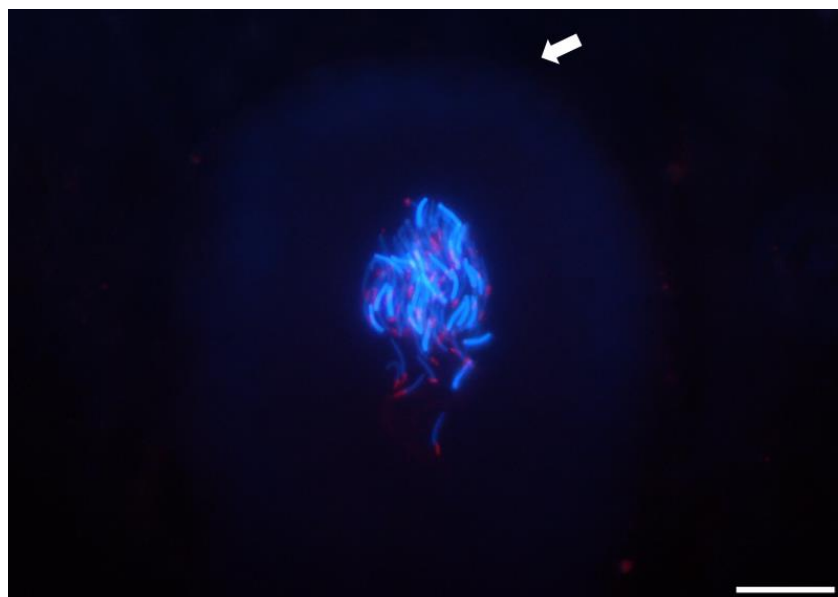




Figure 5: Photomicrograph of a SST of Houbara Bustard (*Chlamydotis undulata*) female containing red and blue stained sperm following the insemination with a mix of sperm. Sperm stained with BLUE have a bright blue nucleus while sperm stained with MRED have a bright red midpiece and a faded blue nucleus; Magnification $\times 400$, merged blue and red fluorescence, scale bar represents $20\mu\text{m}$ and white arrow indicates the dead end of the SST.

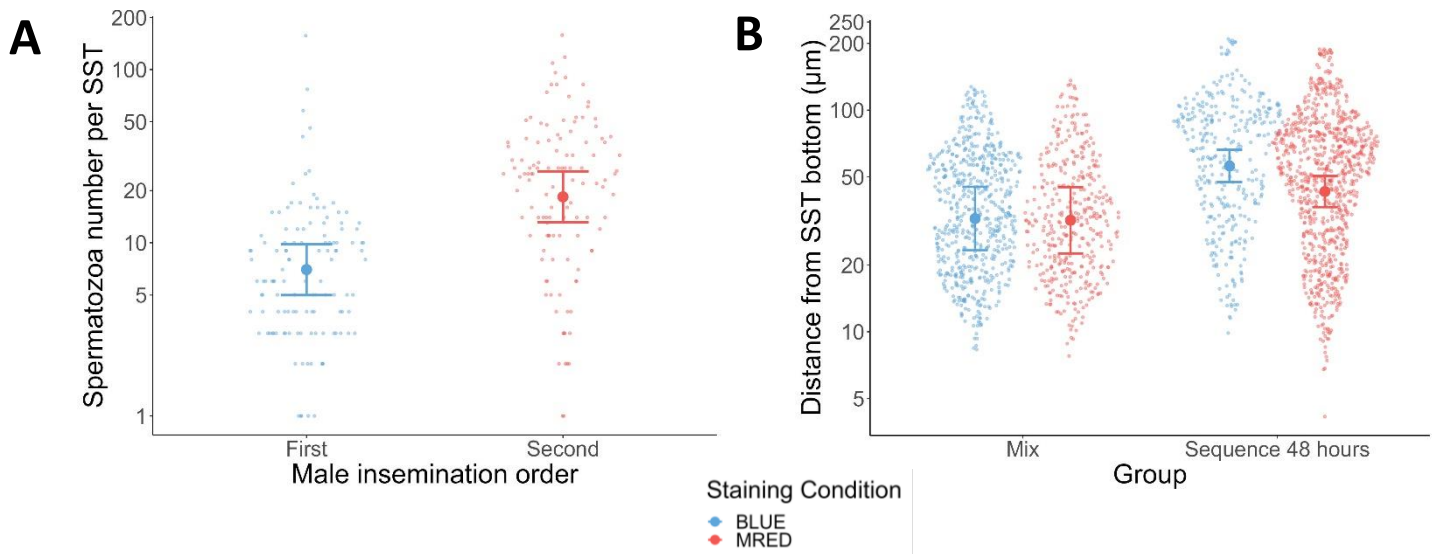


Figure 6: Number of sperm counted per SST according to the insemination order (first insemination = sperm stained with BLUE, second insemination = sperm stained with MRED, with 48 h interval between inseminations) (A). Values are plotted on a log10 scale y axis and expressed as raw data (smaller dots), least-square means (larger dots) and lower and upper 95% confidence intervals (lower and upper error bars) (N = 2 females and 129 SSTs). (B) Distance in μm from the dead end of SSTs of stained sperm of Houbara Bustard (*Chlamydotis undulata*) females inseminated with mixes and in sequence. Values are plotted on a log10 scale y axis and expressed as raw data (smaller dots), least-square means (larger dots) and lower and upper 95% confidence intervals (lower and upper error bars) for each insemination group (N=3 females, 30 SSTs, $n_{\text{BLUE_sperm}}=597$, and $n_{\text{MRED_sperm}}=383$ for Mix group, 2 females and 20 SSTs, $n_{\text{BLUE_sperm}}=338$, and $n_{\text{MRED_sperm}}=861$ for sequence group).

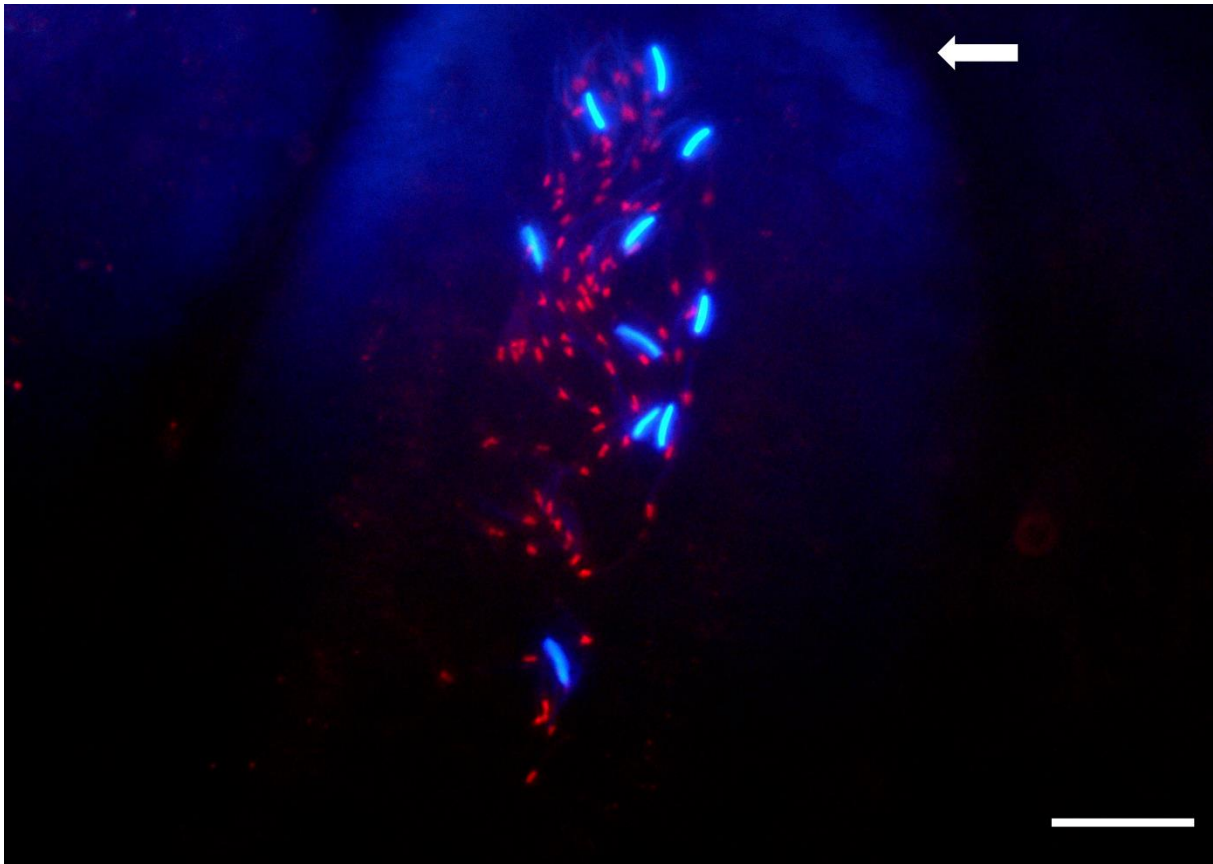
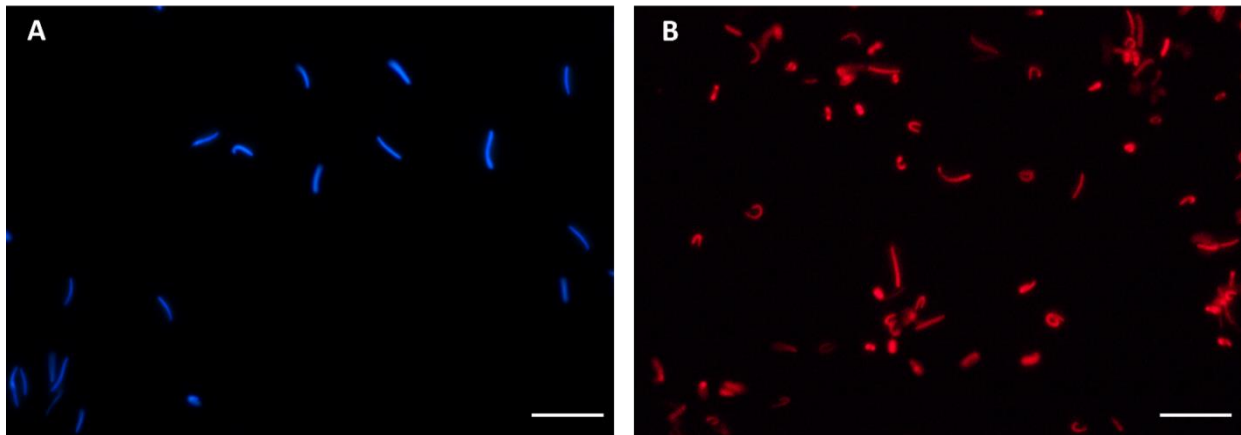


Figure 7: Photomicrograph of an SST of a female first inseminated with BLUE and then with MRED stained sperm (48 hours interval between inseminations) illustrating the higher number of sperm from the second insemination. Magnification $\times 400$, merged blue and red fluorescence, scale bar represents $20\mu\text{m}$ and white arrow indicates the dead end of the SST.

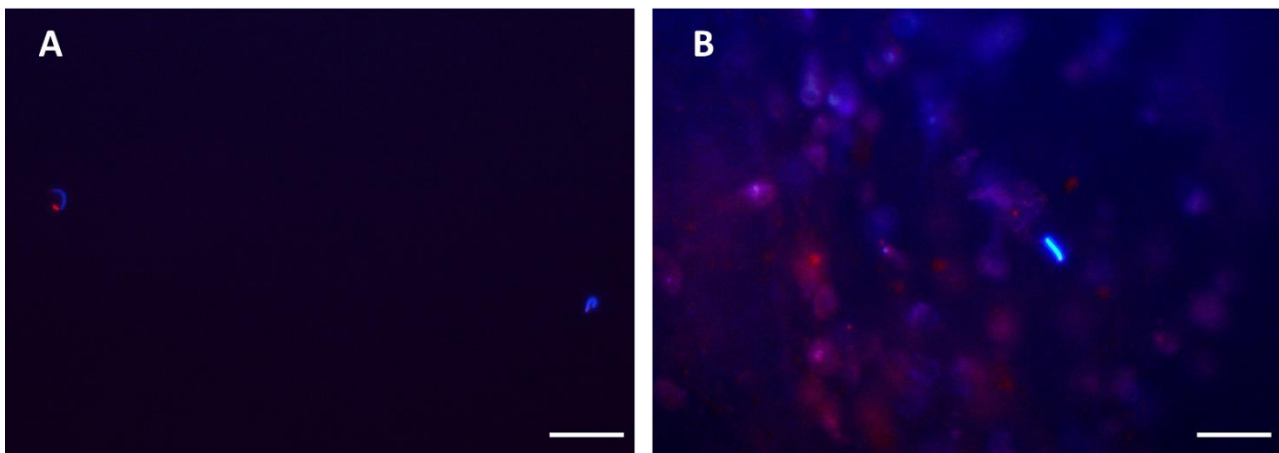
Supplementary Table 1: SCA settings used for the analysis of sperm motility and velocity

a) Microscope		Capture	
Calibration name	10x	Analysis timeout	15
Calibration value ($\mu\text{m}/\text{pixel}$)	0,420918	Box size (pixels)	152
Capture method	Ph-	Check image	yes
Grid distance (μm)	100	Frame rate (fps)	50
Disposable		Number of images	25
Disposable	Proiser	Preview	one second
Depth (μm)	20	Resolution	Low
SS correction factor	1.3	Safe Mode	yes
		Style	Automatic

Parameters		Finish	
Area (μm^2)(min)	5	Sperm to analyze	500
Area (μm^2)(max)	190	Fields to analyze	30
Drifting ($\mu\text{m/s}$)	0	Minimum sperm to analyze	200
Static ($\mu\text{m/s}$) <	10	Minimum fields to analyze	3
Slow-Medium ($\mu\text{m/s}$)	50	Warning (%)	20
Rapid ($\mu\text{m/s}$) >	100		
Progressive (STR>)	70		
Connectivity (pixels)	18		
VAP points (pixels)	5		
VCL/VAP	VCL		



Supplementary Figure 1: Photomicrographs of North African Houbara Bustard (*Chlamydotis undulata*) sperm stained with BLUE (A) and with NRED (B) at 20 μ M concentration after 72 hours of incubation at 40°C. Magnification \times 400, observed with blue and red fluorescence filters, scale bars represents 20 μ m.



Supplementary Figure 2: Photomicrograph of BLUE and MRED stained sperm (A) and embryonic cells (B) on the perivitelline membrane of an egg found inside the uterus of a North African Houbara Bustard (*Chlamydotis undulata*) female inseminated with a mix of sperm; Magnification \times 400, merged blue and red fluorescence, scale bars represent 20 μ m.



CHAPTER 3: TESTOSTERONE RISE PRECEDES THE PEAKS OF SEXUAL DISPLAY AND HIGHER SPERM QUALITY IN ADULTS AND OLD CAPTIVE NORTH AFRICAN HOUBARA BUSTARDS (*CHLAMIDOTYS UNDULATA UNDULATA*)

Lena Meunier^{1,2}, Hiba Abi Hussein¹, Gabriele Sorci², Yves Hingrat¹, Michel Saint-Jalme¹, Janaina Torres Carreira¹

¹Reneco International Wildlife Consultants LLC, Abu Dhabi, P.O.Box 61741, United Arab Emirates.

²Biogéosciences, UMR 6282 CNRS, Université de Bourgogne Franche-Comté, Dijon, France.

* Correspondence: jtcarreira@reneco.org

Manuscript in preparation for submission

Abstract

The North African Houbara Bustard (*Chlamidotys undulata undulata*) is a wild bird species with seasonal reproduction, where males begin displaying well in advance of any eggs laid by the females and display long after the termination of laying. Testosterone is expected to enhance male reproductive display and other sexual signals, therefore, high levels of circulating testosterone may have partly evolved as a physiological basis for high levels of sexual display in polyandric species. At the same time, testosterone is key for maintenance of spermatogenesis. To investigate the relation of testosterone levels across the season with male reproductive behaviour and semen parameters in two distinct age groups, 11 young adults North African houbara bustards (4 to 6 years) and 11 old (14 to 16 years) were evaluated for testosterone levels, semen quality (motility score and sperm number) and behaviour. Testosterone curve peak advanced sperm quality increase and mating behaviour intensification, indicating a possible relation to male-to-male interaction and gonadal preparation for optimal spermatogenesis and sperm output.



Introduction

Seasonal regulated reproduction is a critical aspect of an organism's life history strategy, and many species have evolved mechanisms to synchronize their reproductive activities with optimal environmental conditions, enhancing their reproductive success. Animals, particularly birds, often rely on environmental cues such as photoperiod, rainfall, and food availability to regulate the hypothalamic-pituitary-gonadal (HPG) axis, a key component of the reproductive endocrine system (Watts & Hahn 2012). Studies on birds exposed to natural light conditions and variations in weather have provided valuable insights into the ecological factors influencing testosterone regulation.

Testosterone is an important behaviour modulator as it can influence positively certain reproductive behaviours (like collaborative display and male aggression (Vernasco *et al.* 2020; Lopes *et al.* 2021) and negatively others, like paternal care (Villavicencio *et al.* 2014; Lopes *et al.* 2021). Thus, testosterone effects have been extensively explored due to the extremely variable effects on sexual display, aggressivity (Lopes *et al.* 2021), territory determination (Ryder *et al.* 2011; Lipshutz & Rosvall 2021) song production (Apfelbeck *et al.* 2017) and paternal care (Lopes *et al.* 2021), nevertheless all this parameters will depend on mating system, nesting (Lipshutz & Rosvall 2021), latitude and species (Hau *et al.* 2008; Apfelbeck *et al.* 2017; Moore *et al.* 2019; Lopes *et al.* 2021).

Additionally, besides reproductive behaviour effects, testosterone also influence the expression of secondary sexual traits. These traits can be indicators of male quality and therefore influence female choice (Ditchkoff *et al.* 2001; Pilastro *et al.* 2002; Vergara & Fargallo 2011; Polak *et al.* 2021; Chiara *et al.* 2022). Testosterone is involved in ornaments colouration and size in birds. High levels of testosterone have been shown to increase plumage coloration by regulating carotenoid and melanin pigmentation (Blas *et al.* 2006; McGraw 2008; Lindsay *et al.* 2011; Hiyama *et al.* 2018; Green *et al.* 2022; Khalil *et al.* 2023). Testosterone is also associated with comb size, resulting in increased male's attractiveness, and higher mating success (Ligon *et al.* 1990; Verhulst *et al.* 1999; Rintamaki 2000).

Furthermore, the balance between testosterone production and feedback systems on the hypothalamus-pituitary-gonadal axis determines sperm production and regulation. The pituitary gland releases gonadotropins, FSH and LH, in response to GnRH pulses secreted by the GnRH neurons in the hypothalamus. FSH promotes the differentiation of Sertoli cells, stimulating spermatogenesis (Vizcarra *et al.* 2022). These cells support spermatogenesis by maintaining spermatogonia renewal and providing growth factors and nutrients to developing germ cells (Smith & Walker 2014). LH stimulates the production of androgens by Leydig cells (Vizcarra *et al.* 2022). Testosterone diffuses and binds to androgen receptors of Sertoli cells, initiating cellular signalling pathways necessary for



spermatogenesis activity (Schlatt & Ehmcke 2014; Smith & Walker 2014). Testosterone also regulates LH and FSH by exerting a feedback action on GnRH production (Schlatt & Ehmcke 2014).

The North African Houbara Bustard is a long-lived bird that exhibits a seasonal breeding pattern from January to July (Saint Jaime *et al.* 1996). Concerns about the declining wild population led to the initiation of a captive breeding program in the 1990s, with the species being classified as vulnerable on the IUCN Red List in 2016 (2016). Factors contributing to this decline include the species' high value as a traditional quarry for falconers, poaching, gun hunting, and habitat degradation (Goriup 1997).

While in the wild, male houbara bustards engage in elaborate courtship displays to attract mates, despite females exhibiting promiscuous mating behaviour with multiple sires per clutch (Gaucher *et al.* 1996; Lesobre *et al.* 2010), in captive breeding, the display behaviour is preserved and semen is collected using a dummy female, though reproduction is followed by artificial insemination (Saint Jalme *et al.* 1994).

As previously stated, the role of testosterone in male reproductive success is well-documented, as it enhances displays and sexual signals associated with high mating success (Ball *et al.* 2002; Westneat & Stewart 2003). At the same time, sperm production, regulated by reproductive hormones including testosterone, plays a crucial role in sperm competition, with larger sperm reserves providing a competitive advantage. Comparative studies across species have shown that intense sperm competition correlates with larger testes and increased testosterone production, suggesting a complex interplay between these factors (Birkhead & MØLLER 1993; Moller & Briskie 1995). In a captive breeding context, the role of testosterone levels on pre-copulatory competition maybe not be so evident, but in a context of multiple inseminations it might play a role on sperm production, collection success and post-copulatory sperm competition.

Recent research on houbara bustards indicates that male age negatively impacts ejaculate quality, hatching success, offspring growth and survival, and sperm production (Preston *et al.* 2015; Rabier *et al.* 2021; Meunier *et al.* 2022). Furthermore, older males face reduced fertilization success during competitive interactions with younger males, highlighting the importance of age-specific investment in reproductive traits (Vuarin *et al.* 2019c).

Taking advantage of the birds available at the captive breeding, where samples can be easily collected, behaviour, and semen quality are already routinely recorded for North African houbara bustards, our investigation aims to explore the intricate relationships between testosterone, sperm production, and reproductive behaviours in adults and old male houbara bustards, offering insights into reproductive ageing and the processes shaping their reproductive strategies.



Materials and Methods

Adult (n=11, aged 4 to 6 years) and old (n=11, aged 14 to 16 years) male North African houbara bustards (*Chlamydotis undulata undulata*) were randomly selected from the breeding flock at the Emirates Center for Wildlife Propagation (ECWP) in eastern Morocco. ECWP operates a pre-emptive conservation breeding program aimed at reinforcing natural populations of the North African Houbara Bustard (Lacroix 2003a). The birds were housed in individual outdoor cages (4 sqM) and maintained on a consistent diet throughout the year, comprising commercial 14% protein pellets, water *ad libitum*, ferret pellets (5g), mealworms (10g), and alfalfa (20g).

We closely monitored various reproductive and behavioural parameters as the breeding program relies entirely on artificial inseminations (Saint Jalme *et al.* 1994), including semen collection, evaluation, and dilution. From December 2021 to July 2022, we evaluated testosterone blood levels biweekly over a 7-month period. Simultaneously, we recorded male training performance scores (ranging from 1 to 5), daily displaying behaviour (yes/no), and sperm count and motility score as proxies for semen quality as part of the captive breeding program. It is important to note that only data from 7 young males were usable for semen quality, as 4 did not produce samples.

To determine the peak period and temporal pattern of each of the studied parameter, all the available datasets collected throughout the 2022 breeding season were used. However, for assessing hormonal influences on the observed traits in houbara birds, we considered a specific subset of data; since blood and sperm samples were not always collected on the same day, this subset included records of sperm collection and behavioural monitoring, each matched with testosterone measurements taken within a +/- 3-day window to ensure the relevance and accuracy of any potential hormonal effect.

Two methods were employed to analyse displaying data: (i) comparing the number of displaying versus non-displaying males per day and age group, to discern the pattern of displaying probability across the population during the season; (ii) examining the frequency of displaying during the 14 days preceding the T4 measurement at individual level, to assess the hormonal influences on displaying behaviour.

Serum testosterone

Blood was collected by venipuncture from the jugular vein every other week, always on Thursdays and two hours after the sunrise, from December 2021 until June 2022. Males' collection order was kept throughout all the experiment. After serum separation samples were frozen at -18° C until further analysis. Serum testosterone concentration was analysed using a commercial ELISA kit (ENZO Testosterone ELISA kit, Enzo Life science, Lausen, Switzerland, ADI-901-065) according to



manufacturer's instructions in a portable Absorbance 96 Plate Reader (Enzo-Bionoy, Lausen, Switzerland). This kit has been validated for many bird species (Wada *et al.* 2007; Watts & Hahn 2012; Deviche *et al.* 2016; Alward *et al.* 2016; Watts *et al.* 2016). The inter-assay CV was 10% and intra-assay CV 5.1%.

Display, semen collection success and semen evaluation

Display frequency was recorded once a day from December 2021 to July 2022, trained technicians observe birds daily at the same time and record if the male was displaying during the passage or not (0 or 1). Semen was collected routinely for the breeding management of the center, briefly, males were exposed to a dummy female and its behaviour was scored (bird training score): 0, the bird was stressed; 1, the bird was not interested by neither the dummy nor the keeper; 2, the bird was interested by the dummy and approaches; 3, the bird was interested by the dummy and courtships it; 4, the bird copulated but did not ejaculate; 5, the bird copulated and ejaculated. When an ejaculate was collected, it was immediately transferred to the laboratory where the sample was evaluated for motility index (0-5) and sperm number (concentration, by spectrophotometry/ejaculate volume) (Vuarin *et al.* 2019a).

Statistical analysis

In this study, generalized mixed models were built, considering male identity as a random effect to account for the non-independence of repeated measurements on the same individual, collection day which is designated hereafter by day of the year (DOY) to assess seasonal trends (1= January 1st), and bird age class as a fixed effect. The T hormone data were analysed using a Gaussian regression model with logarithmic transformation, incorporating male weight as an additional covariate due to its known influence on T levels. Displaying probabilities were modelled using a binomial distribution while the number of sperm in the ejaculate was modelled using a Student's t-distribution to accommodate biologically valid outliers. The ejaculate motility score and training performance score were analysed using Cumulative Link Mixed Models (CLMM), suitable for ordinal data. When CLMM models failed to meet the proportional odds assumption, a scale factor was included for non-constant variance across different levels of predictors. Model selection was performed using a backward selection process based on Bayesian Information Criterion (BIC) and Akaike Information Criterion (AIC). Multicollinearity was regularly assessed, and residual plots were examined for model diagnostics. Graphical representations of model predictions were generated based on the marginal means of the data. This comprehensive analysis was conducted using R statistical software, utilizing packages including dplyr, glmmTMB, lubridate, DHARMA, ggeffects, and ordinal.



Ethical statement

The breeding program runs under the approval of the Moroccan Ministry of Agriculture and a mandate independent veterinarian (mandate number: 534–98), providing control of the wellbeing of the birds. Onsite veterinary facilities ensure the best possible care for sick or injured birds by a team of expert veterinarians, and standards from sanitary authorities are regularly controlled.

Results

Based on raw data, old and adult males exhibited similar means (+/-standard deviations, SD) across the different studied parameters (Table 1). Furthermore, models showed no statistically significant effect of age group on all parameters except for the training scores (Table 2 and Figure 1); CLMM model revealed a lower training performance of young males compared to old males. Young males also tended to have a higher sperm count in their ejaculates compared to older males, although this difference was not statistically significant.

Additionally, although a 10% decrease in weight was observed throughout the season, it did not significantly affect testosterone levels.

All models identified a significant impact of day of the year (DOY) on the various studied parameters highlighting clear seasonal fluctuations throughout the observed period. Each parameter values increased gradually at the onset of the study to reach a peak level, and then gradually declined. Figure 2 offers a comprehensive comparison of these seasonal patterns, displaying per DOY: (i) the testosterone level; (ii) the likelihood of obtaining a training score of 5 (%); (iii) the daily percentage of displaying males; (iv) the likelihood of having a sperm collection with a motility score above 3 - the standard minimum threshold used for insemination (%); (v) and the sperm count in the ejaculate ($\times 10^6$). Models revealed asynchronized peaks between testosterone levels and reproduction and sexual behavioural parameters, as shown in Figure 2. Testosterone concentrations in the blood reached its maximum level around DOY 50, and subsequently begin to decline gradually thereafter. However, with a similar trend to testosterone, the displaying probability and number of sperm in the ejaculate peaked almost 25 to 30 days later; this delay suggests a possible long term hormonal impact on the houbara sexual behaviour or a catalyst role of testosterone in reproductive performance. Training performance, on the other hand, reached its maximum by day 80 of the year; the linear increase in the first 80 days, suggests a continuous improvement or accumulation of skills that houbara bustards acquire over time. Sperm motility, however, shows a different pattern, peaking considerably later in the season, around day 125.

While testing for potential hormonal influence of T on reproduction and behavioural traits on our restricted dataset, significant effects were observed for sperm motility and training performances



only (Table 3). Figure 3 shows that the probability of collections with low motility scores (1.5 or 2.5) remains relatively stable regardless of testosterone blood concentrations. A slight increase in the likelihood of getting a low score was identified for T levels higher than 7500 pg/mL. In contrast, high motility scores (3.5 and 4) show a marked increase in probability with rising testosterone levels, peaking at a moderate range between 2000 and 5000 pg/mL, before declining at higher T concentrations. Similarly, Figure 4 reveals a clear trend where training performance tends to improve as testosterone levels increase, until reaching an optimal range. For instance, a top performance score of 5 is achieved when T levels hovers between 4500 and 7500 pg/mL. Beyond this range, the probability of maintaining a high score decreases considerably.

Discussion

The findings revealed non-simultaneous peaks of the studied parameters, the peak of testosterone precedes the peaks of sexual display, collection success and higher sperm quantity and motility, that we can simplistically define from now as higher sperm quality, suggesting the hormonal rise to be necessary as a preparation for breeding. In a review on the relations between hormones and behaviour, Wingfield et al. (2001) (Wingfield *et al.* 2001) shown that captive birds (Song sparrows, *Melospiza melodia morphna*) had a testosterone rise before the first brood and that after the concentrations reduced until the end of the season. This group also described that this pattern was different for wild birds of the same species, explaining they had to establish territories and interact with other males, what they called a challenging effect (Wingfield *et al.* 2001), that would be more evident and present in monogamous species, while for polygamous, male to male interactions were claimed to not be related to testosterone peaks (Wingfield *et al.* 2001). In the wild, houbara bustards will display in sites separated by an average distance of 550 meters, forming what's known as exploded leks (Cornec *et al.* 2017).

In this experiment, male North African houbara bustards were kept in individual cages, which did not completely remove the possibility of males watching others' sexual displays and hearing the specific booming sounds produced by other males. These booms are deep vocalizations that play a crucial role in their courtship displays in the wild (Cornec *et al.* 2017).

The results indicate a rise of testosterone before the increase of sexual display and training score 5, thus shows that for this species testosterone rises marking the establishment of the reproductive season and is relevant for the initiation of sexual behaviour, possibly playing a role in male interaction. On a related species, the great bustard, Biczó and Péczely (2007) (Biczó & Péczely 2007) observed two rises in testosterone levels in captivity, one concurrent peak of testosterone and full display with ejaculation, but also a previous rise of T level was found at the early phase of the season, in a positive



correlation with the increasing number of aggressive male-male interactions. The same group found that in great bustards testosterone levels may correlate with traits determining the quality of male displays (colour intensity of feathers, number of moustachial feathers, and size of gola) (Biczó & Péczely 2007).

The first peak of testosterone in male houbara could correspond to the establishment of territories in wild populations. Later, the medium level of testosterone recorded over the breeding season could be sufficient to maintain courtship behaviour and spermatogenesis. The energetic cost of displaying could explain the decreased body mass during the breeding season. Previous studies, have shown that males displayed for several hours each day, and high plasma levels of testosterone and the resulting increase in activity associated with displays may be energetically expensive (Saint Jaime *et al.* 1996).

The impact patterns of testosterone on motility and training performance indicate that optimal scores for both traits correlate with a specific testosterone range. Thus, on the other hand, detrimental effects of very high testosterone levels are seen in both sexual traits.

However, from a statistical point of view, the analysis was conducted with a small sample size of only 22 individuals; this represents a major challenge, especially in terms of statistical power and findings generalization. Furthermore, the necessity of maintaining short intervals between blood and sperm collection restricted even more our dataset size. Those two main challenges impacted mainly the results of the analysis of T impact on the studied traits and might explain: (i) the inability to find a significant direct link between testosterone and all the sexual traits; (ii) the wide confidence intervals in our results, which suggest a high degree of uncertainty and potential variability in the effects among individuals. Despite those challenges, the trend of testosterone impacts on motility and performance scores were consistent, even on our small dataset, which lends credibility and strength to our results. Our results showed that probability of high sperm motility score was associated with moderate levels of testosterone, in a range between 2000 and 5000 pg/mL. Similarly, in zebra finches, a recent study reported a positive covariation between testosterone levels and sperm motility and velocity (Hurley *et al.* 2023). However, we also showed that when testosterone reached a high concentration, over 7500 pg/mL, the risk of low motility score increased. This suggests a trade-off between sperm motility and testosterone concentration over a certain threshold. Males with very high testosterone would have lower sperm motility compared to males producing a normal range of testosterone. To our knowledge such trade-offs were never reported in avian species. However, (Rudolfson *et al.* 2006) reported in the Arctic Charr (*Salvelinus alpinus*), an external fertilizer teleost, that dominant males showed higher testosterone concentrations and lower sperm velocity compared to subdominant ones, suggesting that males exhibiting high testosterone levels have impaired sperm quality.



Food, light duration, and weather features were equal for all males, so the analysed parameters variations were not influenced by this characteristic. Although older males have been collected through all their lives and all individuals were born in captivity and trained for semen collection equally during their first 2 years of life, meaning older males had more years of interaction than young adults, what could be the reason for higher training scores.

However, trying to trace a correspondence to the wild we have to consider that amongst lekking species, houbara bustards presents one of the highest levels of polyandry with 60 % of multiple paternities (Lesobre *et al.* 2010), thus we could think that young males could compensate lower display experience, or territory disadvantage, with higher sperm number or global ejaculate quality (Preston *et al.* 2015; Vuarin *et al.* 2019c; Meunier *et al.* 2022).

Conclusion

The study presents a comprehensive investigation into the relationship between testosterone, sperm production, and reproductive behaviour in adult and old male captive North African houbara bustards under natural light and weather conditions. The results reveal interesting patterns, showing that peaks of the studied traits were not simultaneous. Notably, the peak of testosterone precedes the peaks of sexual behaviour and reproductive traits. This sequence may suggest that hormonal peaks are necessary for houbara birds to be physiologically ready for breeding.

In addition to the physiological aspects, research on bird testosterone also plays a role on conservation biology. Investigations into endangered species, such as the North African Houbara Bustard, can bring light to the endocrine mechanisms underlying reproductive success and help to improve captive breeding and conservation strategies.



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Figures

Table 1: Summary table reporting the mean and the standard deviation per age group based on the raw data of the studied parameters.

Parameter	Group	Mean	Standard deviation
Testosterone level (T, pg/mL)	Young (N=153)	3723.6	2485.5
	Old (N=149)	3813.6	2082.5
Sperm motility score	Young (N=249)	3.16	1.05
	Old (N=399)	2.96	0.91
Number of sperm (*10 ⁶)	Young (N=249)	17.18	14.61
	Old (N=399)	11.27	9.95
Male training score	Young (N=909)	3.07	1.41
	Old (N=884)	3.93	1.33
Males daily displaying percentage	Young (N=230)	21.90	18.28
	Old (N=230)	21.78	12.87

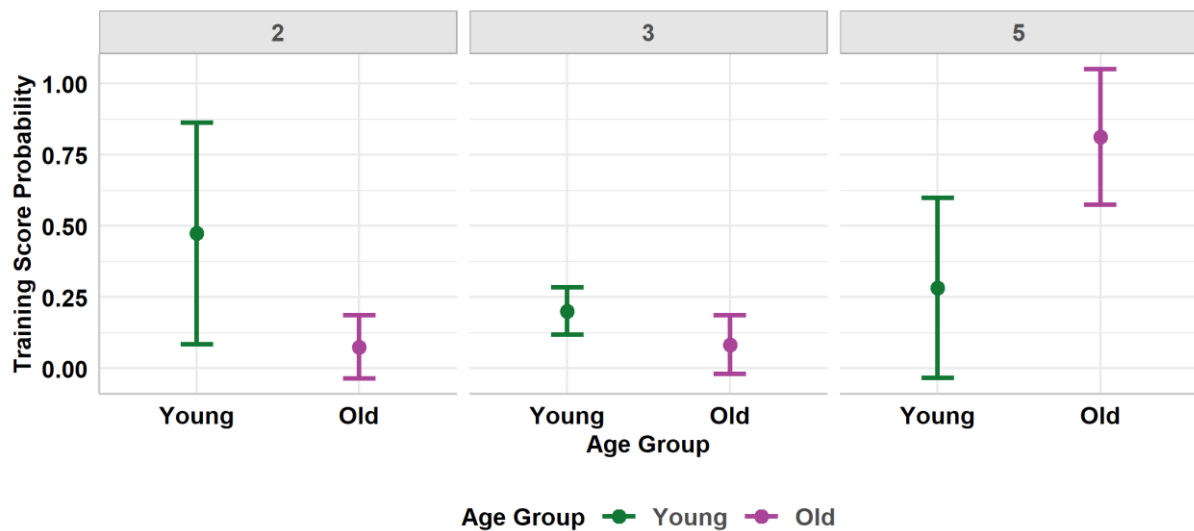


Figure 1: Predicted probabilities of male training score per Age group. Predictions for scores 1 and 4 were not shown since they had almost a null occurrence in our dataset.

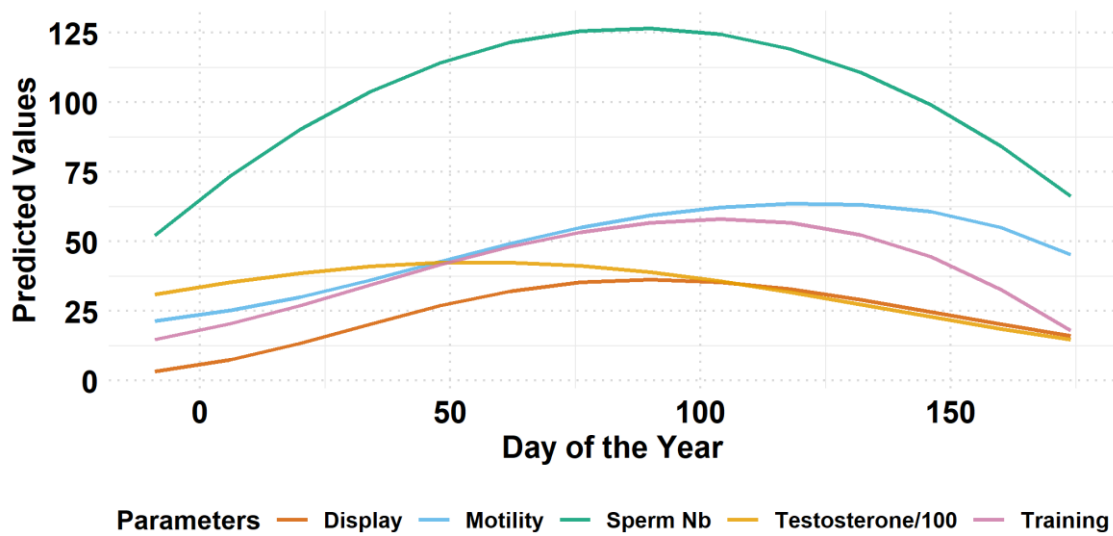


Figure 2: Predicted seasonal trends of Testosterone levels, the probability of displaying, sperm count and motility, and training performance of houbara males. January 1st was set as the reference point. Dates falling in December of the previous year, are represented with negative values. Testosterone level (pg/mL*0.01) in green; the likelihood of obtaining a training score of 5 in yellow (%); the daily percentage of displaying males in grey; the likelihood of having a sperm collection with a motility score above 3 in blue (%); (v) and the sperm count in the ejaculate in red (*10⁶).

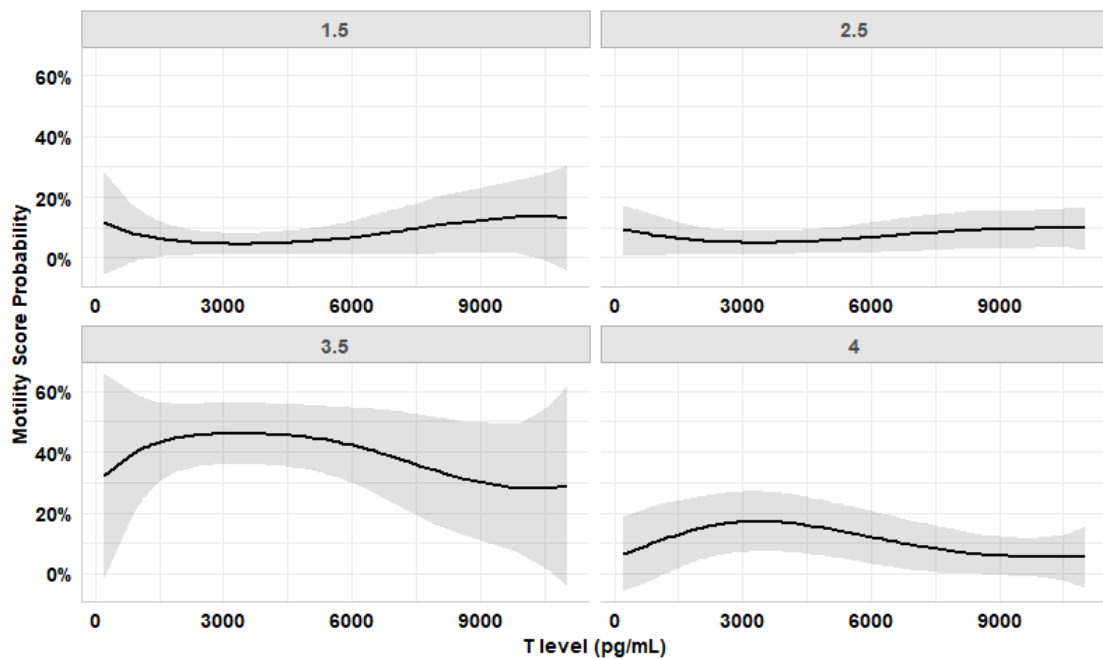


Figure 3: Impact of Testosterone level on the most occurrent motility scores in our restricted dataset.

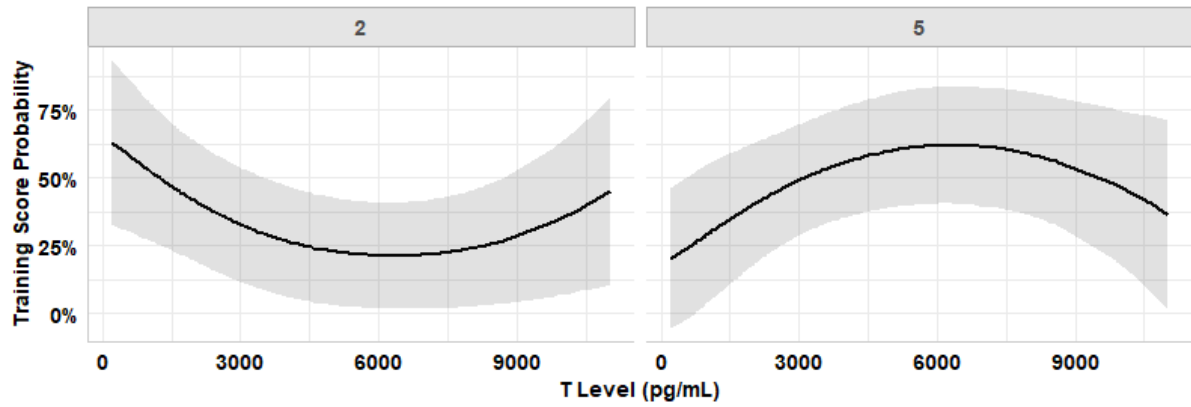


Figure 4: Impact of Testosterone level on the most occurrent training performance scores in our restricted dataset.

Table 2: summary of final models assessing the impact of age group and day of the year (DOY) on the studied parameters.

Parameter	Variable	Estimate	95% CI	P-Value	
Log(T)	Intercept	8.05	7.90, 8.20	<0.001	
	DOY	-4.07	-4.95, -3.19	<0.001	
	DOY ²	-3.77	-4.65, -2.89	<0.001	
	Random: Male_ID	0.34	0.24, 0.47	-	
Probability of displaying	Intercept	-1.45	-1.53, -1.37	<0.001	
	DOY	3.36	1.25, 5.47	0.002	
	DOY ²	-16.83	-18.79, -14.89	<0.001	
	DOY ³	3.61	1.72, 5.50	<0.001	
Number of sperm (*10⁶)	Intercept	10.97	8.35, 13.58	<0.001	
	DOY	6.74	-8.18, 21.66	0.376	
	DOY ²	-49.42	-64.61, -34.23	<0.001	
	Random: Male_ID	5.49	3.88, 7.77	-	
Sperm motility score	DOY	11.66	7.3, 16	<0.001	
	DOY ²	-8.36	-13, -4.2	<0.001	
	DOY ³	-4.38	-8.5, -0.30	0.035	
	Random: Male_ID	1.26	0.89, 1.87	-	
	<i>Scale parameters</i>				
	DOY	0.75	-3.6, 5.1	0.4669	
	DOY ²	2.47	-1.7, -6.7	0.016	
	DOY ³	1.95	-2.1, 6.0	0.056	
	Male training score	DOY	21.75	17, 27	<0.001
		DOY ²	-33.02	-39, -27	<0.001
DOY ³		-12.31	-18, -6.4	<0.001	
Age Group - Young		-2.83	-5.1, -0.56	0.015	
Random: Male_ID		2.66	1.96, 3.82	-	
<i>Scale parameters</i>					
DOY		3.98	-1.4, -9.4	0.15	
DOY ²		4.26	0.70, 7.8	0.019	

T= Testosterone; CI = Confidence Interval; DOY = Day of the year.

Parameter	Variable	Estimate	95% CI	P-Value	
Sperm motility score	DOY	5.25	0.70, 9.8	0.024	
	DOY ²	-4.59	-8.3, -0.85	0.016	
	T	-3.14	-6.7, 0.44	0.085	
	T ²	-4.51	-8.1, -0.93	0.013	
	T ³	3.26	-0.43, 7.0	0.083	
	Random: Male_ID	1.06	0.62, 1.76	-	
	<i>Scale parameters</i>				
		DOY	4.74	1.7, 7.8	0.002
		DOY ²	-3.52	-7.0, -0.06	0.046
		DOY ³	5.28	2.6, 8.0	<0.001
Male training score	DOY	17.46	11, 24	<0.001	
	DOY ²	-9.07	-15, -3.0	0.003	
	DOY ³	-6.34	-11, -1.2	0.015	
	Age Group - Young	-2.80	-5.4, -0.19	0.035	
	T	7.75	0.37, 15	0.039	
	T ²	-6.40	-15, 1.7	0.123	
	Random: Male_ID	2.86	1.92, 4.46	-	
	<i>Scale parameters</i>				
		T	-2.53	-9.9, 4.8	0.15
	T ²	6.30	-1.8, 14	0.001	

T= Testosterone; CI = Confidence Interval; DOY = Day of the year.

Supplementary material

Table 3: Summary of the parsimonious regression mixed models of T4 levels and number of sperm in the ejaculate.

Characteristic	T4 Levels			Number of Sperm		
	Estimate	95% CI ¹	p-value	Estimate	95% CI ¹	p-value
Day of the year						
Day of the year	0.02	0.01, 0.04	<0.001	6.7	-8.2, 22	0.4
Day of the year ²	0.02	0.01, 0.05	<0.001	-49	-65, -34	<0.001

¹ CI = Confidence Interval

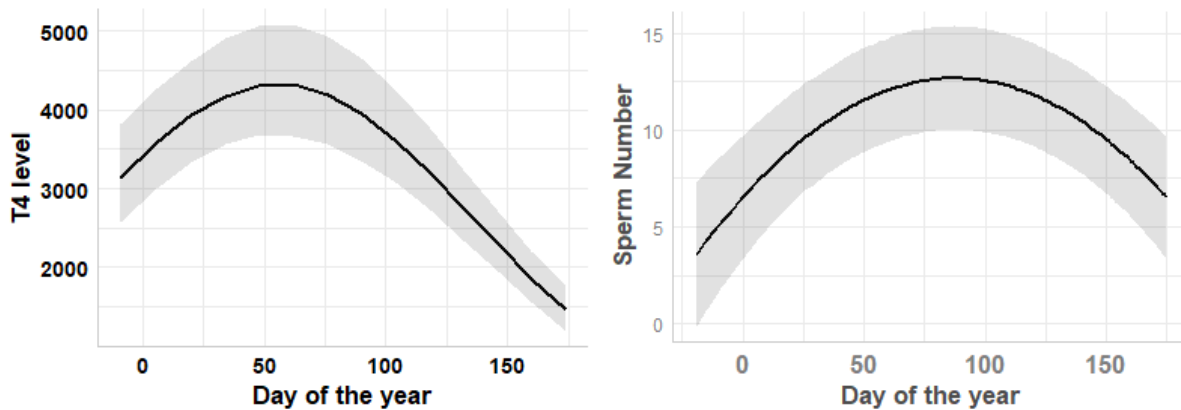


Figure S. 1: Impact of date of collection on T4 Levels and number of sperm in the ejaculate. January 1st was set as the reference point. Dates prior to January 1st, falling in December of the previous year, are represented with negative day.



CHAPTER 4: PHENOTYPIC TRADE-OFFS BETWEEN SPERM QUANTITY AND QUALITY ACROSS AGE GROUPS IN A POLYANDROUS BIRD, THE NORTH AFRICAN HOUBARA BUSTARD

Léna Meunier^{1,2*}, Janaina Torres Carreira¹, Caroline Silva Vieira^{1,3}, Yves Hingrat¹, Michel Saint Jalme¹, Gabriele Sorci²

¹ Reneco International Wildlife Consultants LLC, Abu Dhabi, United Arab Emirates

² Biogéosciences, UMR 6282 CNRS, Université de Bourgogne, Dijon, France

³ Federal University of Minas Gerais, UFMG, Belo Horizonte-MG, Brazil

*corresponding author, lmeunier@reneco.org

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Abstract

When females mate with several males during the same reproductive bout, male siring success depends on the relative numbers of competing sperm and their quality. Therefore, when the risk of sperm competition is high, males should invest more in both sperm quantity and quality. However, due to energetic costs or other constraints (e.g., replication errors during cell divisions), sperm quantity and quality are usually predicted to trade against each other. Here, we assessed the pattern of covariation between sperm quantity and quality across age groups in a bird species with a lek-based mating system, undergoing strong post-copulatory sexual selection, the North African Houbara Bustard. Contrary to the prediction, we did not find evidence supporting the hypothesis of a trade-off between sperm quantity and quality. Rather, males that produced large ejaculates (high sperm numbers) also produced sperm of high quality in terms of motility, velocity, or normal morphology. Moreover, the strength of the positive covariation between sperm quantity and quality did not depend on male age (similar slopes between sperm quantity and quality in prime age and senescent males). The only evidence suggesting a trade-off between sperm quantity and quality refers to the relationship between ejaculate size and the DNA fragmentation index since ejaculates containing large number of sperm also had high proportions of sperm with fragmented DNA. We discuss the possible reasons underlying the discrepancy between the predicted trade-off and the observed pattern of positive covariation. In particular, we advocate for the need of estimating genetic correlations between sperm quantity and quality.



Introduction

In polyandrous species, females mate with multiple males during the same reproductive bout (Taylor et al. 2014). Multiple mating extends the opportunity for sexual selection to operate after copulation has occurred, as sperm from multiple males can compete for egg fertilization (Birkhead and Pizzari 2002). Sperm competition has been found to be a strong component of post-copulatory sexual selection, shaping investment in ejaculate and sperm attributes (Simmons and Fitzpatrick 2012; Fitzpatrick and Lüpold 2014; Macartney et al. 2024). Both inter- and intraspecific analyses have highlighted the importance of sperm competition in shaping the investment in sperm quantity and quality (Gage and Morrow 2003; Garcia-Gonzalez and Simmons 2005; Fitzpatrick et al. 2009). For example, comparative studies have shown that males have larger testes in species with a promiscuous mating system (Lüpold et al 2020). Similar results have been reported within species where males can adjust their investment in ejaculates in terms of quantity or quality of sperm produced, depending on the perceived risk of sperm competition (delBarco-Trillo and Ferkin 2006; Thomas and Simmons 2007; Bartlett et al. 2017).

In competitive matings, the probability that a given male's sperm will succeed in fertilizing the egg is likely to depend on a combination of different factors. All other things being equal, the most obvious characteristic that could potentially explain differential fertilization success between males is the number of sperm transferred to the female. This is the principle of fair raffle where the greater the number of tickets we can buy, the greater the chance of winning the prize (Parker 1990). As mentioned above, this principle works under the restrictive condition that all other traits potentially involved in the fertilization process do not vary between competing males. However, this is highly unlikely to occur, as several ejaculate and sperm characteristics have been identified as important determinants of fertilization success, including sperm viability, motility, velocity and morphology (equivalent to a loaded raffle) (Parker and Pizzari 2010). Therefore, in an ideal world, males facing high risk of sperm competition should simultaneously invest in different aspects of the ejaculate to maximize both the quantity and quality of sperm produced.

However, a different outcome is often observed for several reasons. First, errors in the replication of the genetic material can occur during cell division. The male germline is particularly at risk of accumulating replication errors due to the number of divisions required to produce functional gametes and indeed males typically have higher mutation rates in the germline than females (Ellegren 2007; Venn et al. 2014). Therefore, if these replication errors are not repaired, or DNA integrity is impaired, we might expect that ejaculates containing higher numbers of sperm would also accumulate higher proportions of sperm with morphological or genetic defects. Second, the production of large ejaculates containing high quality sperm may incur significant energetic costs. For example, Thomsen



et al. (2006) estimated that the ejaculates produced daily by male Japanese macaques (*Macaca fuscata*) can account for up to 6% of the basal metabolic rate. There is also considerable evidence to support the idea that ejaculate characteristics are condition-dependent, as shown by studies that have manipulated nutrient intake (Macartney et al. 2019), although some sperm characteristics appear to be more sensitive to individual condition than others. Condition dependence paves the way for strategic allocation of resources to different aspects of ejaculate and sperm function, resulting in trade-offs between them.

One of the most studied trade-offs is the one between sperm number and size. As mentioned above, one of the simplest ways to increase fertilization success under competitive conditions is to produce more sperm. This can be achieved by allocating more resources to sperm production (e.g., larger testes) or by reducing sperm size. Although in many species ejaculates consist of large numbers of relatively small sperm, in some cases ejaculates can contain few but very large sperm (Immler et al. 2011; Zajitschek et al. 2019). Some studies have also reported positive selection on sperm size (Bennison et al. 2015), although this relationship does not appear to be consistent across species (Macartney et al. 2024). Selection on sperm size is thought to occur due to the faster swimming speed of sperm with a long flagellum or long midpiece, two structures important for sperm propulsion (Bennison et al. 2015). Therefore, there are *a priori* reasons to expect a trade-off between sperm number and velocity.

From this brief overview, it is clear that maximizing different traits of the ejaculate may be difficult to achieve due to the inherent constraints linking different components. Despite these theoretical expectations, it is not uncommon to find reports of positive covariation between quantity and quality (Kortet et al. 2004; Malo et al. 2005; Lüpold 2013; Malmsten et al. 2015). Interspecific evidence of positive covariation between sperm quantity and quality may reflect differences in the relative risk of sperm competition between species (Lüpold 2013). At the intraspecific level, other mechanisms could be at play, such as a high variance in energy acquisition between individuals (van Noordwijk and de Jong 1986). For example, senescent individuals may have fewer resources to allocate to the ejaculate compared to males at prime age (Lemaître et al. 2020), and therefore studies reporting positive correlations between sperm quantity and quality without controlling for the confounding effect of age may simply reflect inter-individual differences in resource acquisition. Alternatively, ageing may exacerbate the strength of the trade-off between sperm traits. Old individuals suffer from increased oxidative stress (Lemaître et al. 2020). Old males who invest more in sperm number may therefore have less energy left for proper germline maintenance, which should result in a stronger trade-off between quantity and quality compared to young individuals.

Here, we conducted a study in which ejaculates of male North African houbara bustards (*Clamydotis undulata undulata*) were characterized for a number of traits describing different



components of sperm quality and used to examine the pattern of covariation with investment in ejaculate size (number of sperm in the ejaculate). We analysed sperm of birds of prime and senescent age, allowing us to estimate the covariation independently of age, and to assess whether age increases the strength of the expected trade-off between sperm quantity and quality. The North African Houbara Bustard is a polyandrous species in which females mate with multiple males (Lesobre et al. 2010). Males have a peculiar sexual display at specific sites (exploded leks) and are subject to strong post-copulatory sexual selection (Hingrat et al. 2005; Vuarin et al. 2019). The birds used in this study are part of an *ex-situ* conservation program where birds are bred under captive conditions with the aim of restoring declining natural populations across the species range of the species (Lacroix 2003).

Based on the rationale described above, we expected to find a trade-off between sperm quantity and quality in both age classes. We also predicted that the slope of the relationship between sperm quantity and quality should be steeper for senescent individuals than for prime age birds.

Material and Methods

Animals

All birds used for this experiment were part of a conservation breeding program [Emirates Center for Wildlife Propagation (ECWP)] of the North African Houbara Bustard located in Missouri (Morocco). Birds, males and females, were reared in outdoor pens and received *ad libitum* food (commercial specially formulated dry food pellets, mealworms, and crickets) and water.

Ejaculate collection

A total of 454 ejaculates were collected from 171 males divided in 2 groups: senescing males from 14 to 20 years old ($n = 71$, mean \pm SD = 16.10 ± 2.06 years) and young males from 3 to 5 years old ($n = 100$, mean \pm SD = 3.84 ± 0.80 years). Ejaculates were collected in the morning by a trained ECWP staff member, using a dummy female. After displaying, the male mounted the dummy and started copulating. A glass Petri dish was used to collect the ejaculate by pressing it gently on the male cloaca. The ejaculate was immediately transferred to a 2 mL cryotube. Ejaculates were rapidly evaluated for volume and number of sperm and diluted (1:10) in PBS-BSA (1.5%). Samples were maintained at room temperature until analysis (less than one hour).

Sperm number

The number of sperm in the ejaculate was assessed using a spectrophotometer (600nm, Jenway 6051, Cole-Parmer, USA). The optic density was transformed in sperm concentration with a specific calibration previously set for the species (Chargé et al. 2010), and the concentration value was divided by ejaculation volume to obtain total sperm number.



Sperm quality traits

Sperm quality was assessed using a series of traits describing different functional aspects of the cell fertilization potential: 1) sperm motility (percentage of motile sperm in the ejaculate); 2) curvilinear velocity of motile sperm ($\mu\text{m}/\text{sec}$); 3) progressive sperm (percentage of sperm with progressive movement in the ejaculate); 4) mitochondrial membrane potential (percentage of sperm with polarized mitochondrial membrane in the ejaculate); 5) DNA fragmentation index (DFI%, percentage of sperm with fragmented DNA in the ejaculate); 6) High DNA stainability (HDS%, percentage of sperm with decondensed DNA in the ejaculate); 7) viability (percentage of sperm with intact membrane in the ejaculate); 8) sperm morphology (percentage of sperm with normal morphology in the ejaculate); 9) teratogenic sperm (percentage of sperm with teratogenic morphological defects in the ejaculate); 10) ejaculate volume (μl); 11) concentration of round cells in the ejaculates ($10^6/\text{ml}$).

Flow cytometry analyses

Sperm viability, mitochondrial potential, DFI% and HDS% were measured by flow cytometry using a Guava easycyte 5HT (IMV, L'Aigle, France) flow cytometer equipped with a 150-mW blue argon laser (488 nm) and 3 colour photomultipliers green (525/30 nm), yellow (583/26 nm) and red (695/50nm). For each sample, 10,000 events were counted in the sperm population gate. Data were analysed using the guavaSoft IMV software 4.0 (IMV, L'Aigle, 131 France).

Viability

The viability or membrane integrity was assessed for 425 ejaculates of 163 males using Easy Kit™ 1 viability (IMV, L'Aigle, 131 France), which contains propidium iodide (PI) and SYBR-14 dye dried and pre-loaded in 96 rounds bottoms well plates. SYBR-14 dye is membrane permeable and sperm emitting green fluorescence at 518 nm when bound to nucleic acids (Garner et al. 1994). PI enters only damaged plasma membranes and binds to nucleic acids and when excited at 488nm emits red fluorescence at 617 nm (Gillan et al. 2005). Twenty microliters of diluted (1:10) sample were transferred in the kit plate with 180 μL of PBS/BSA 1.5% and mixed. Negative control was made by incubating 20 μl of samples mix with 180 μL of Triton X 1% in 1.5mL cryotubes for 15 minutes in the dark at room temperature. Triton X is a detergent that permeabilizes the membranes. Samples were washed by centrifugation for 5 minutes at 1000g and resuspended in 180 μL of PBS/BSA 1.5%. Finally, 200 μL of the treated samples were loaded to the 96 wells kit plate and mixed. Samples were analysed after 15 minutes of incubation in the dark at RT on a plate shaker.

Mitochondrial potential

Mitochondrial potential was assessed for 418 ejaculates of 162 males using EasyKit™ 2 Mitochondrial activity (IMV, L'Aigle, 131 France), which contains JC-1 dye dried and pre-loaded in 96 rounds bottoms well plates. JC-1 fluorochrome accumulates in monomer form in depolarized



mitochondria emitting green fluorescence at 529 nm. In response to high mitochondrial membrane potential, monomers aggregate and emit orange fluorescence at 590 nm. First JC1-1 dye was resuspended by adding 5 μL of DMSO and mixing. Then 20 μL of diluted (1:10) sample were diluted in 180 μL of PBS/BSA 1.5% and transferred to the kit plate. For positive control, 0.25 μL of Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) (50 mM, from MitoProbe™ JC-1 Assay Kit (Molecular Probes, USA) were added to 200 μL of diluted (1:10) sample, incubated 5 minutes at 37°C and transferred in the kit plate. CCCP is a mitochondrial membrane potential disrupter that inhibits oxidative phosphorylation and thus provokes depolarization of mitochondrial membranes. Samples were analysed after 30 minutes of incubation in the dark at RT on a plate shaker.

DNA fragmentation and condensation

The DNA fragmentation and compaction was measured for 408 ejaculates of 162 males with the Sperm Chromatin Structure Assay (SCSA) with modifications (Evenson and Jost 2000). This technique consists in a metachromatic stain, acridine orange (AO), to intercalates into double-strand DNA and emits green fluorescence (515–530 BP filter) and stacks on single-strand DNA and emits red (630 LP filter) upon excitation from a 488 nm laser, The extent of single and double DNA strand breaks (DNA fragmentation index, %DFI) and level of excess nuclear histones (high DNA stainable sperm, %HDS) are simultaneously measured in individual sperm (Evenson, 2022).

A phosphate-citrate buffer was prepared by adding 370 ml of citric acid buffer (0.1M) and 8.77g of NaCl to 630 ml of Na₂PO₄ buffer (0.2M) and then stored at 4°C. An AO staining solution (3 $\mu\text{g}/\text{ml}$) was made with AO stock solution (Sigma, 1mg/ml) and the phosphate-citrate buffer. In a 96 well plate, 180 μL of AO staining solution was added in each well with 20 μL of diluted semen (1:10 in PBS/BSA 1.5%). For HDS positive control, to permeabilize the membrane and reduce protamine disulfides (Simões et al. 2009, Szczygieal and Ward 2002), 20 μL of control sample were incubated with 180 μL of dithiothreitol (DTT) 2nM/ Triton 0.5% for 10 minutes in the dark on a plate shaker. After incubation, the sample was centrifuged for 5 minutes at 1000g, resuspended in 180 μL of AO solution and 200 μL were transferred in the plate. Samples were analysed after 3 minutes of incubation in the dark at RT on a plate shaker.

Motility

Sperm motility parameters were measured for 438 ejaculates of 168 males with Sperm Class Analyzer (SCA) software (Microptic). Four microliters of diluted semen (1:20 in PBS/BSA 1.5% for most samples, 1:10 or 1:40 if the concentration was too low or too high) were placed on 20 μm depth disposable chamber (4 chambers/slide, Proiser, ISAD4C20) under a negative phase contrast microscope (Nikon Eclipse E200, Japan, 10x objective) linked to the computer by a camera. A maximum of 30 fields were analysed as to reach 500 sperm (see Table S1 in the supplementary material for details



on SCA settings). Percentage of total motile, progressive and immotile sperm were counted, velocity and trajectory parameters were recorded as well as the concentration of round cells (millions/ml).

Morphology

Sperm morphology was assessed for 414 ejaculates of 168 males. Fixed solutions of semen were first prepared by adding 5 μ L of 4% buffered paraformaldehyde (40% PFA diluted 1:10 in PBS 1X) to 10 μ L of diluted ejaculate (1:10). Then 6 μ L of the fixed solution were placed on a slide, covered with a coverslip and observed under a phase contrast microscope (Nikon Eclipse E200, Japan, objective x100, oil immersion). For each sample, 100 sperm cells were observed, and each classified in 8 categories (Bloom 1973; Alkan et al. 2002): acrosome loss, cytoplasmic droplet, swollen head, head defect, midpiece defect, tail defect, teratogenic sperm and normal sperm cell. Samples with very low concentration were not assessed for sperm morphology as it was not possible to count 100 cells.

Statistical analyses

Ejaculate sperm number, mitochondrial membrane potential, DFI%, HDS%, ejaculate volume, and round cell concentration were log-transformed to reduce skewness. Covariation between traits was assessed using Pearson's correlation coefficients.

The trade-off between sperm quantity and quality was modelled using general linear mixed models (GLMMs) with a normal distribution of errors. In these models, the log-transformed number of sperm in the ejaculate was the dependent variable and the different characteristics describing sperm quality were included as fixed terms (one variable per model). Age class and the interaction between age class and sperm quality were also included as fixed effects. Male ID was included as a random factor as some males contributed multiple ejaculates. Degrees of freedom were estimated using the Satterwaite approximation.

As we considered 11 traits describing different functional aspects of sperm quality, we also performed a principal component analysis (PCA) to reduce the dimension of the multivariate space. The traits were standardised (mean = 0, std = 1) before performing the PCA. A threshold of 10% of explained variance was used to extract the most important components ($n = 4$). These components were then used together as independent variables in a GLMM with the same structure as described above. All analyses were performed in SAS (9.4).

Ethical statement

The breeding program runs under the approval of the Moroccan Ministry of Agriculture and a mandate independent veterinarian (mandate number: 534-98), providing control of the well-being of the birds. Onsite veterinary facilities ensure the best possible care for sick or injured birds by a team of expert veterinarians, and standards from sanitary authorities are regularly controlled. Experiments



are conducted ethically, and each breeding protocol is developed while ensuring appropriate and ethical treatment of birds. To minimize stress during capture, birds are fitted with hoods during manipulation. Facilities are designed to ensure the well-being of the birds and prevent injuries.

Results

The correlation matrix containing the 12 traits (one related to sperm quantity and 11 related to sperm quality) included in our analysis showed that the correlation coefficients ranged from -0.65 (viability vs. high DNA stainability) to 0.835 (percentage of progressive sperm in the ejaculate vs. VCL) (Table S2 in the supplementary material). Covariation was the highest for traits related to presumably linked functions, showing that our panel of traits consistently captured different aspects of sperm/ejaculate quality. For example, ejaculates with high proportions of sperm with polarized mitochondrial membrane were expected to contain high proportions of motile sperm, and this was indeed the case ($r = 0.414$) (Table S2 in the supplementary material). Similarly, DNA decondensation (HDS%) and fragmentation (DFI%) were expected to be negatively correlated with sperm viability, and indeed the proportion of live sperm in the ejaculate decreased as long as the proportion of sperm with decondensed DNA increased ($r = -0.65$) (Table S2 in the supplementary material). Figure 1 shows the correlation matrix as a heat map, allowing the relative strength and sign of the covariations to be visualised. Mean values, standard deviations, minimum and maximum of each sperm traits are reported in Supplementary table 1.

We then ran eleven GLMMs with the number of sperm in the ejaculate as the response variable and each of the sperm quality characteristics as an independent variable. These models also tested the effect of male age and the interaction between male age and sperm quality. Of the 11 relationships tested, only three had the expected sign under the hypothesis of a trade-off between sperm quantity and quality, and of these, only one was statistically significant (Table 1). Ejaculates with large numbers of sperm also contained higher proportions of sperm with fragmented DNA (Figure 2a). Visual inspection of Figure 2a showed that some points were outside the main cloud of data. We therefore reran the model excluding these points in order to see if the statistical significance depended on their inclusion. This model showed that removing these data strengthened the relationship ($\beta = 0.197$, 95% CI = 0.096-0.299, $p < 0.0001$; Figure 2b). The majority of sperm quality traits were positively and significantly correlated with sperm number (Table 1), showing that for most functional aspects, these birds were able to invest in large ejaculates and also produced high quality sperm.

Birds of prime age consistently produced more sperm in their ejaculate in all GLMMs (Table 1). However, the interaction between sperm quality and age was never significant, except for the model including ejaculate volume, where the positive slope relating sperm number to ejaculate volume was



slightly steeper for old birds (Table 1). These results suggest that the relationship between sperm quantity and quality is remarkably constant from prime to senescent age.

The PCA including the 11 quality traits reduced the dimensions of the sperm quality space to 4 principal components. The principal components 1 to 4 explained 25%, 15%, 12% and 11% of the variance, respectively. Component 1 had positive loadings for percentage of motile sperm, percentage of progressive sperm, VCL, percentage of viable sperm in the ejaculate, percentage of sperm with a normal morphology, and negative loadings for DNA fragmentation index and high DNA stainability (Figure 3). Component 2 mainly captured the negative relationship between viability (negative loadings) and high DNA stainability (positive loadings) (Figure 3). Component 3 captured the negative relationship between morphologically normal sperm (negative loadings) and teratogenic sperm (positive loadings) (Figure 3). Finally, component 4 had positive loadings for the percentage of sperm with polarized mitochondrial membrane and the concentration of round cells in the ejaculate, and negative loadings for ejaculate volume (Figure 3).

The GLMM with the number of the sperm in the ejaculate as the dependent variable and the four principal components as fixed effects (plus age class and the interaction between the principal components and age class) showed that components 1, 2 and 4 were positively and significantly associated with sperm number (Table 2, Figure 4). Component 3 was not associated with sperm number (Table 2, Figure 4). None of the age class by component interactions were statistically significant, supporting the view that age does not alter the overall relationship between sperm quantity and sperm quality.

The correlation matrix between sperm quality traits (Table S2 in the supplementary material) showed moderate to high coefficients (> 0.5) for two trait combinations (sperm viability vs. high DNA stainability; percentage of progressive sperm in the ejaculate vs. VCL). We therefore ran two additional GLMMs to test the statistical significance of these relationships and to assess possible differences in the slopes between age classes. The percentage of viable sperm was generally very high $93.46\% \pm 5.57\%$ ($n = 163$ males, 425 ejaculates); however, ejaculates with the highest percentage of sperm with decondensed DNA also had the lowest percentage of viable sperm (Table 3, Figure 5A). This result confirms the functional role of the high DNA stainability index as a potential marker of sperm quality in the North African Houbara bustard. Consistent with the results on the relationship between sperm quantity and quality, age did not alter the slope of the relationship between viability and high DNA stainability (Table 3, Figure 5A). The other pair of traits (VCL and the percentage of progressive sperm) were very strongly positively correlated, with similar slopes for the two age classes (Table 3, Figure 5B).



Discussion

Contrary to our prediction, we found that sperm quantity and quality positively covary in males of the North African Houbara bustard, and this covariation is similar across a large range of ages (prime vs senescent). The only evidence suggesting that allocation to sperm number might compromise one aspect of sperm quality regards the proportion of sperm with fragmented DNA. Again, male age did not change the strength of this trade-off.

Males of the North African Houbara Bustard (*Chlamydotis undulata undulata*) have a peculiar sexual display expressed in dedicated sites (exploded leks) during the breeding season (Hingrat et al. 2005). Females visit males and can mate with several males as revealed by high levels of clutches with multiple paternities (Lesobre et al. 2010). Females can also store sperm in specific structures (sperm storage tubules) where sperm stay viable for several weeks as shown by females laying fertile eggs more than 40 days post-insemination (Sorci et al. 2021). Therefore, multiple mating and sperm storage set the scene for episodes of post-copulatory selection affecting among male variation in reproductive success.

In species under strong post-copulatory selection, we expect males to invest more in traits increasing the likelihood of egg fertilization, both in terms of quantity and quality. However, due to energetic costs and inherent constraints operating on different aspects of sperm quality, it might be impossible to simultaneously produce large ejaculates containing high quality sperm, resulting in a trade-off between sperm quantity and quality. Recent work has emphasized how the investment into the maintenance of the germline can be costly (Chen et al. 2020) and that selection for increased sperm production incurs a cost in terms of higher risk of de novo mutations that might have deleterious effects on the progeny (Blumenstiel 2007; Wong 2014; Baur and Berger 2020). We did not assess the frequency of mutations in ejaculates containing large or small number of sperm, but we found that large ejaculates did contain a higher proportion of sperm with fragmented DNA. The DNA fragmentation index showed a skewed right distribution with a few samples having quite high values compared to the bulk of the samples. However, a subsequent model that excluded these outliers confirmed that the higher the number of sperm in the ejaculate the higher the proportion of them with fragmented DNA. The integrity of DNA is key for the proper functioning of sperm, to achieve the fertilization of the ovum and contribute to embryo development (Lewis and Aitken 2005). The causes leading to sperm DNA damage are manifold, and include, for instance, single and double strand breaks produced by oxidative stress (Sakkas and Alvarez 2010). Sperm are particularly vulnerable to oxidative stress for several reasons (Birch et al. 2024). Sperm are metabolic active and have membranes (polyunsaturated fatty acids) susceptible to oxidative attacks. They also have reduced antioxidant capacity, which may be due to the limited cytoplasmic volume or other specific reasons related to



seasonality and use of resources to reestablish testicular function (Birch et al. 2024). Protection against oxidative stress is nevertheless provided by antioxidant enzymes contained in the seminal fluid. The protection of the germline is however costly, as shown by recent work on different model systems (Chen et al. 2020; Koppik et al. 2023). In particular, there is evidence suggesting that senescing individuals are more prone to oxidative damage due to their impaired capacity to produce or acquire antioxidant defenses (Lemaitre et al. 2020). Here, we found that sperm DNA fragmentation was higher in ejaculates containing higher number of sperm, corroborating the idea that higher investment into sperm production comes with a cost in terms of germline maintenance. This result is in agreement with previous work showing that species and individuals exposed to high levels of sperm competition also have high values of DNA fragmentation. delBarco-Trillo et al. (2016) assessed the amount of DNA fragmentation in 18 species of rodents with different intensity of sperm competition (using relative testis size as a proxy) and found positive correlation between the two traits. Similar results have been reported in the Zebrafish where individuals exposed to high levels of male-male competition had more sperm with DNA damage in the ejaculate (Silva et al. 2019). Interestingly, however, we did not find any evidence suggesting that age might exacerbate the trade-off between sperm quantity and DNA fragmentation. This result therefore does not support the hypothesis that senescing individuals may be less able to face oxidative stress or to fail providing effective protection to the germline, although a more direct assessment of antioxidant defenses in the seminal fluid is needed.

In addition to the DNA fragmentation index, the Sperm Chromatin Structure Assay (SCSA) provides another index related to the degree of nuclear compaction, the High DNA stainability index. The relevance of this index as a functional indicator of sperm quality (e.g., in terms of fertilization capacity) has been questioned (Mohammadi et al. 2020) and recent work suggests that this index should not be used as a marker of sperm DNA damage (Lu 2022). We did not find any correlation between High DNA Stainability and sperm number in the ejaculate; however, we do report a strong correlation between this index and the proportion of live sperm in the ejaculate, the higher the amount of sperm with fragmented DNA the higher the proportion of dead sperm. Therefore, we suggest that this index might contain valuable information at least for the North African Houbara Bustard. Further work should nevertheless elucidate the link between HDS and fertilization under competitive and non-competitive inseminations.

With the exception of the DNA fragmentation index and percentage of round cells none of the other traits assessed as markers of sperm quality did trade against sperm quantity. If any, the pattern of covariation indicated that males able to produce large ejaculates also produced sperm of high quality. This finding is based on a large array of traits known to describe different dimensions of the sperm quality. These dimensions included four traits referring to the capacity of sperm to move (motility, progressivity, velocity and mitochondrial membrane potential), one trait referring to the



sperm viability, two traits referring to the sperm morphology (morphological normal sperm and teratogenic sperm), one integrative trait referring to the volume of the composed ejaculate (seminal fluid and sperm), and one integrative trait referring to the amount of immature sperm and/or inflammatory cells in the ejaculate (concentration of round cells). Despite this rather large spectrum of sperm quality traits, and contrary to our *a priori* predictions, we found significantly positive correlations with sperm number for motility, VCL, progressivity, mitochondrial membrane potential, normal morphology, ejaculate volume and concentration of round cells. Reports of positive covariation between traits predicted to trade against each other are not uncommon (e.g., Malo et al. 2005), and there are several possible explanations for this discrepancy. Van Noordwijk and de Jong (1984) provided a very elegant explanation for positive covariation between life history traits showing that when individuals differ more in their capacity to acquire resources than in their allocation rule to conflicting functions, we should expect positive correlations. This argument can generally account for positive correlations in free-ranging animals that obviously differ in the amount of resources they can gather when living in variable and unpredictable environments. However, this probably does not apply to the case reported here, since males were maintained under homogenous environmental conditions and fed *ad libitum*. Therefore, we consider unlikely that the positive correlations reported here might arise due to huge among individual variance in the amount of available resources.

Another possible explanation for the observed result is that, given that post-copulatory selection does occur in the breeding facility (females can be inseminated with sperm collected from different males), males with high values of different components of sperm quality may have a substantial competitive advantage (Sorci et al. 2023). Thus, despite effort to maximise representativity of breeders, volume and sperm quality traits may represent selective barriers in captivity that are not counterbalanced by environmental selection as expected in the wild.

We think it is necessary to remind a few important limitations of this study and the associated results. First, despite our sample size allowed us to have a large statistical power and to reject the null hypothesis of no correlation between sperm quality and quantity, the effect size was very small. Indeed, a look at the plots clearly showed that data were largely scattered around the model predictions and only a small fraction of the variance in sperm quantity was accounted for sperm quality, even when the sperm quality space was reduced to the principal four dimensions. This suggests that we can only loosely predict sperm quality based on knowledge on sperm quantity. Second, we only assessed phenotypic correlations. Proper assessment of evolutionary trade-offs relies on genetic correlations. If our hypothesis of post-copulatory selection plus relaxed environmental constraints in the breeding facility is correct, we also expect to find positive genetic correlations between sperm quantity and quality. In a previous study, we estimated the genetic variation of ejaculate size and two traits describing sperm quality (viability and normal morphology) (Chargé et al. 2013). This work



showed significant heritability of sperm quantity while estimates of genetic variation for sperm viability and normal morphology were very low and not different from zero. However, it should be mentioned that in Chargé et al. (2013) the proportion of live sperm and morphologically normal sperm was assessed using an eosin–nigrosine method, whereas methods used here might provide more accurate values. Moreover, Chargé et al. (2013) only considered two traits describing sperm quality, therefore future work should aim at estimating evolutionary trade-offs using a multidimensional space as done in the present study. Finally, we only report associations between traits, which does not allow us to conclude about the causal link between them. Experimental approaches are definitely needed to infer the causal relationship between traits, and especially so when individuals are maintained under benign environmental conditions.

To conclude we report evidence for positive covariation between sperm quantity and quality in the North African Houbara Bustard, except for one marker of DNA integrity for which we found the expected trade-off with investment in ejaculate size. We believe these results are of interest for both the understanding of the adaptive response of males experiencing high levels of post-copulatory selection and for the management of the captive flock. In particular, if sperm quality and quantity are phenotypically but also genetically correlated, given that post-copulatory selection does operate in the breeding flock, we should expect that successful males (those siring the offspring during competitive inseminations) are high quality individuals with both high investment into sperm quantity and quality.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author contribution statement

LM, JTC, YH, MSJ, and GS conceived the study; LM, CSV, and JTC collected the data, GS analysed the data and prepared the tables and figures; GS and LM wrote the first draft of the manuscript; all authors edited, revised, and approved the final version.

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Tables

Table 1. General linear mixed models exploring the association between sperm number (log-transformed) and 11 traits describing different aspects of ejaculate/sperm quality. To avoid collinearity, each ejaculate/sperm quality trait was included in a separate model. Independent variables were standardized (mean = 0, std = 1) prior to their inclusion in the GLMMs. The models also included the age class (prime vs old) and the interaction between ejaculate/sperm quality trait and age class. Some males contributed with multiple ejaculates and the male ID was declared as random intercept in the models to take into account the non-independence of values from the same male. We report the parameter estimate for the 11 ejaculate/sperm quality traits with the 95% confidence intervals. Parameter estimates with confidence intervals non-overlapping zero were considered statistically significant. We also report the predicted sign of the relationship according to the trade-off hypothesis (red indicating a discrepancy between the expected and the observed signs, green indicating a consistency between prediction and observation, and white non-significant effects), the effect of age class and the interaction between age class and ejaculate/sperm quality traits (p values), and the sample size (number of males and number of ejaculates). The effect of age class was always consistent with prime age birds having higher number of sperm compared to old ones. The only significant interaction between ejaculate quality and age class showed that old males had a slightly steeper slope between ejaculate size and volume compared to prime age males. The models also included male ID as a random factor and consistently accounted for a significant fraction of variation in the dependent variable (all p's < 0.0001).

<i>Fixed effects</i>	<i>Parameter estimate</i>	<i>95% CI</i>	<i>Expected sign if trade-off</i>	<i>Effect of age class (p values)</i>	<i>Δ in slope between age classes (p values)</i>	<i>N (males, ejaculates)</i>
Sperm motility	0.18	0.06/0.30	-	0.0064	0.6092	168, 438
VCL	0.16	0.05/0.27	-	0.0087	0.7753	168, 438
Viability	-0.02	-0.11/0.08	-	0.0087	0.3170	163, 425
Mitochondrial membrane potential	0.32	0.18/0.45	-	0.0091	0.5020	162, 418
Sperm morphology	0.18	0.06/0.30	-	0.0048	0.5474	164, 414

DNA fragmentation index	0.10	0.01/0.19	+	0.0088	0.2998	161,408
DNA decondensation	0.07	-0.04/0.18	+	0.0091	0.2339	161,408
Progressive sperm	0.14	0.03/0.24	-	0.0070	0.3112	168,438
Ejaculate volume	0.28	0.17/0.38	-	0.0331	0.0275	171,454
Concentration of round cells	0.12	0.02/0.21	+/-	0.0070	0.7756	168,438
Teratogenic sperm	-0.03	-0.16/0.11	+	0.0067	0.6065	164,414



Table 2. General linear mixed model exploring the association between sperm number (log-transformed) and the four most important components extracted from a PCA with the 11 ejaculate/sperm quality traits. The model also included the age class (prime vs old) and the interaction between principal components and age class. Prime age was considered as the reference group, therefore a negative parameter estimate indicates lower ejaculate size in old birds. Some males contributed with multiple ejaculates and the male ID was declared as random intercept to take into account the non-independence of values from the same male. We report the parameter estimate with the 95% confidence intervals, degrees of freedom, F and p values for the fixed effects, and parameter estimate, standard error, Z and p values for the random effect. N = 158 males and 396 ejaculates.

<i>Fixed effects</i>	<i>Parameter estimate</i>	<i>95% CI</i>	<i>df</i>	<i>F</i>	<i>p</i>
Principal component 1	0.117	0.049/0.185	1,382	23.68	<0.0001
Principal component 2	0.221	0.144/0.299	1,368	40.71	<0.0001
Principal component 3	-0.068	-0.172/0.036	1,381	1.68	0.1962
Principal component 4	0.121	0.016/0.227	1,386	13.06	0.0003
Age class (prime)	-0.323	-0.555/-0.091	1,125	7.60	0.0067
Principal component 1 x age class (prime)	-0.005	-0.098/0.088	1,382	0.01	0.9196
Principal component 2 x age class (prime)	-0.056	-0.175/0.063	1,368	0.85	0.3566
Principal component 3 x age class (prime)	0.034	-0.120/0.189	1,381	0.19	0.6645
Principal component 4 x age class (prime)	0.026	-0.120/0.173	1,386	0.13	0.7238
<i>Random effect</i>	<i>parameter estimate</i>	<i>SE</i>		<i>Z</i>	<i>p</i>
Male ID	0.343	0.067		5.12	<0.0001



Table 3. General linear mixed models exploring the association between sperm viability and high DNA stainability (above) and between VCL and the percentage of progressive sperm in the ejaculate (below). The models also included the age class (prime vs old) and the interaction between principal components and age class. Prime age was considered as the reference group, therefore a negative parameter estimate indicates lower ejaculate size in old birds. Some males contributed with multiple ejaculates and the male ID was declared as random intercept to take into account the non-independence of values from the same male. We report the parameter estimate with the 95% confidence intervals, degrees of freedom, F and p values for the fixed effects, and parameter estimate, standard error, Z and p values for the random effect. N = 161 males and 406 ejaculates, and 168 males and 438 ejaculates, respectively.

Dependent variable = Sperm viability					
<i>Fixed effects</i>	<i>Parameter estimate</i>	<i>95% CI</i>	<i>df</i>	<i>F</i>	<i>p</i>
High DNA stainability	-0.654	-0.756/-0.552	1,400	283.5	<0.0001
Age class (prime)	-0.069	-0.233/0.096	1,102	0.69	0.4087
High DNA stainability x age class (prime)	0.099	-0.042/0.240	1,400	1.91	0.168
<i>Random effect</i>	<i>parameter estimate</i>	<i>SE</i>		<i>Z</i>	<i>p</i>
Male ID	0.075	0.035		2.11	0.0174
Dependent variable = VCL					
<i>Fixed effects</i>	<i>Parameter estimate</i>	<i>95% CI</i>	<i>df</i>	<i>F</i>	<i>p</i>
Progressive sperm	0.831	0.766/0.895	1,381	1172.75	<0.0001
Age class (prime)	-0.105	-0.263/0.052	1,138	1.76	0.1872
Progressive sperm x age class (prime)	-0.059	-0.151/0.033	1,381	1.57	0.2107
<i>Random effect</i>	<i>parameter estimate</i>	<i>SE</i>		<i>Z</i>	<i>p</i>
Male ID	0.183	0.031		5.84	<0.0001

Figures

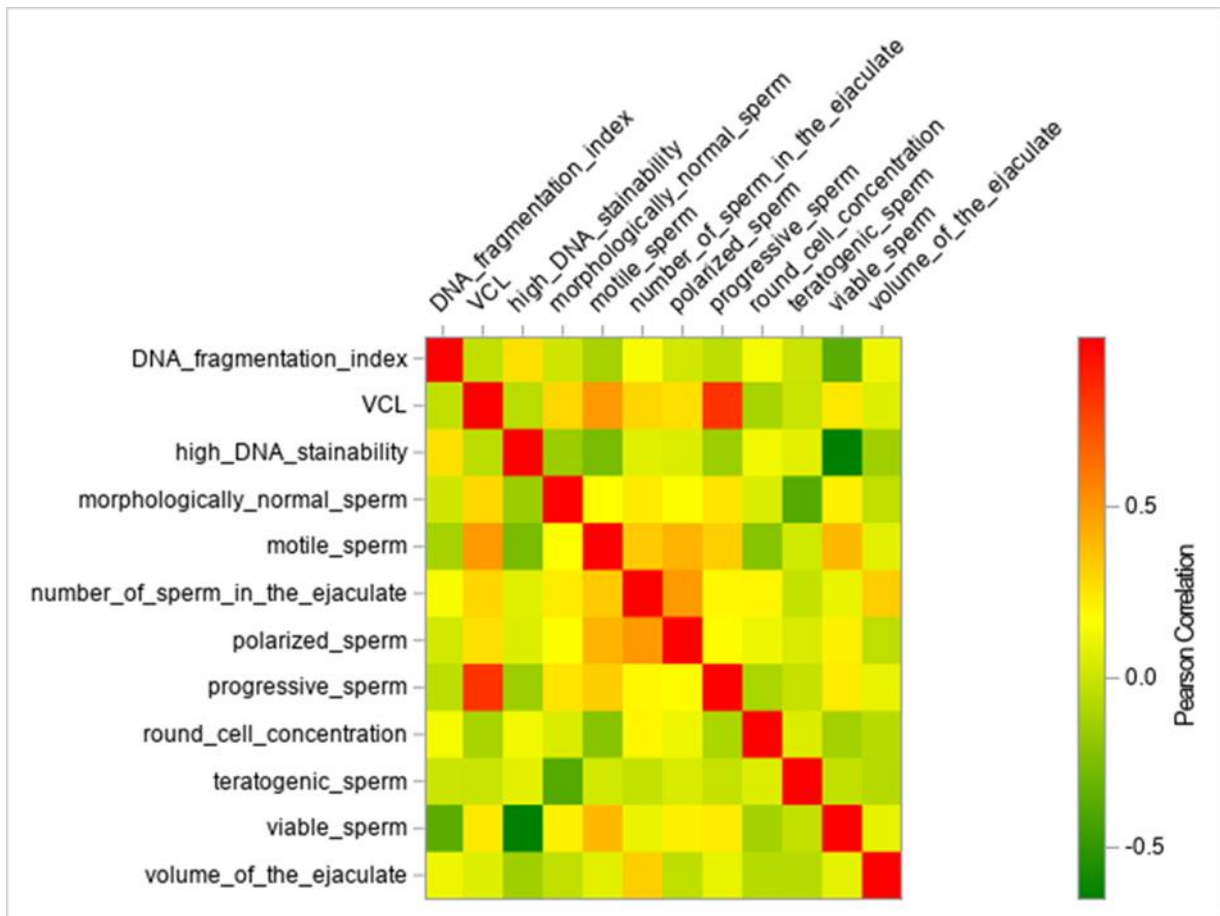


Figure 1. Heatmap illustration the strength and sign of the correlations between the 12 traits included in the analysis. Number of sperm in the ejaculate refers to the investment into sperm quantity, the other 11 traits refer to different aspects of ejaculate/sperm quality. Sample sizes range from 399 to 454 ejaculates.

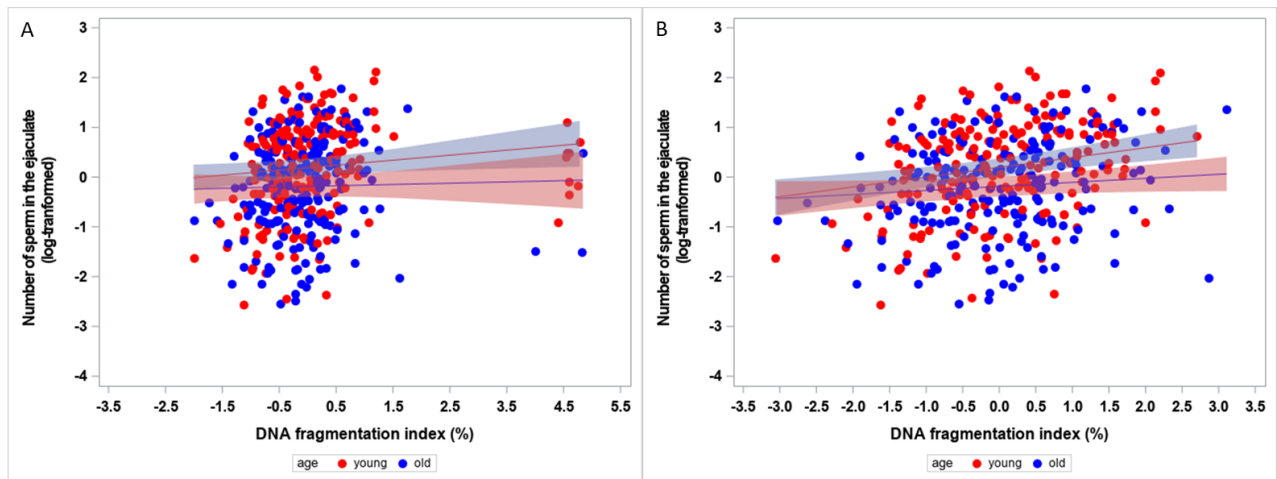


Figure 2. Positive relationship between ejaculate size (the number of sperm in the ejaculate) and the DNA fragmentation index in prime and old male North African houbara bustards. Both variables were log-transformed and standardized. The shaded area corresponds to the 95% CI of the slope. A) represents the relationship considering the whole sample. B) displays the same relationships after exclusion of outliers

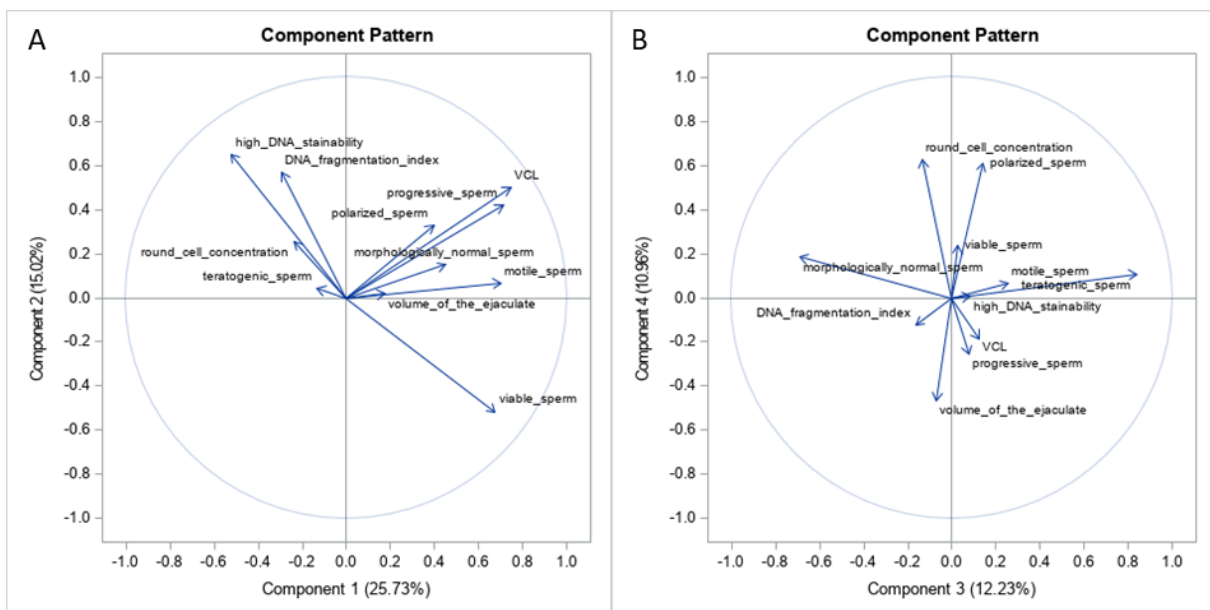


Figure 3. Patterns of loadings for the principal components 1 and 2 (A), and 3 and 4 (B). These components were extracted from a PCA that included the 11 ejaculate/sperm quality traits.

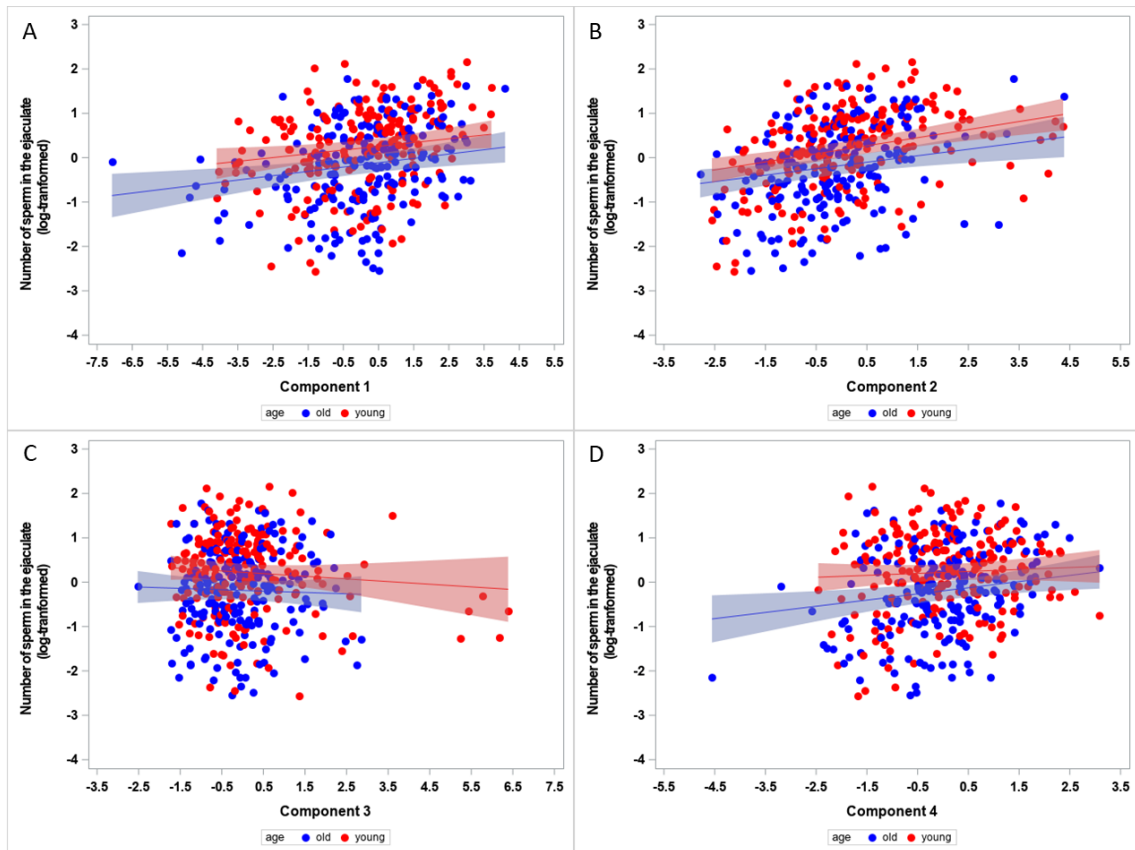


Figure 4. Relationship between ejaculate size (the number of sperm in the ejaculate) and the four components (A to D) extracted from a PCA that included 11 ejaculate/sperm quality traits in prime and old male North African houbara bustards. The shaded area corresponds to the 95% CI of the slope

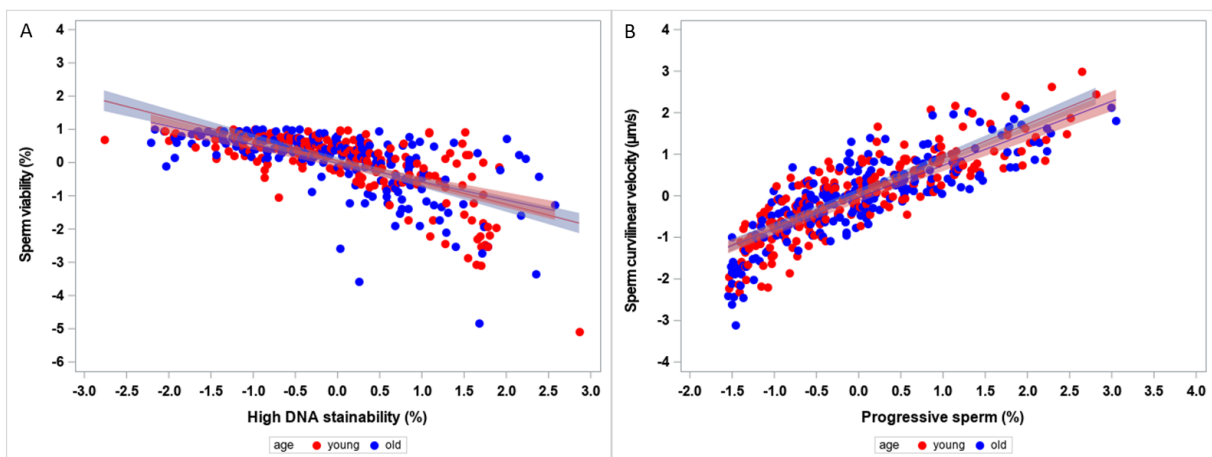


Figure 5. Relationship between sperm viability (the proportion of live sperm in the ejaculate and high DNA stainability (A), and between VCL ($\mu\text{m/s}$) and the percentage of progressive sperm in the ejaculate (B) in prime and old male North African houbara bustards. The shaded area corresponds to the 95% CI of the slope.

Supplementary table I : Mean values, standard deviations, minimum and maximum of the different sperm parameters measured : ejaculate volume ($N_{old}=230 / N_{young}=224$), concentration ($N_{old}=230 / N_{young}=224$), sperm number ($N_{old}=230 / N_{young}=224$), total motile ($N_{old}=223 / N_{young}=215$), progressive percent ($N_{old}=223 / N_{young}=215$), VCL ($N_{old}=223 / N_{young}=215$), viable % ($N_{old}=217 / N_{young}=208$), sperm with polarized mitochondria percent ($N_{old}=213 / N_{young}=205$), percent of sperm with fragmented DNA ($N_{old}=207 / N_{young}=201$), percent of sperm with decondensed DNA ($N_{old}=207 / N_{young}=201$), percent of sperm with normal morphology ($N_{old}=207 / N_{young}=207$) and percent of sperm with teratogenic defect ($N_{old}=207 / N_{young}=207$).

age category	Sperm parameter	Ejaculate Volume (μ l)	Sperm Concentration (Million/ml)	Sperm Number (Million)	Total motile sperm (%)	Progressive (%)	VCL (Avg) (μ m/s)	Viable (%)	Polarized (%)	DFI (%)	HDS (%)	Normal morphology (%)	Teratogenic defect (%)	Round cell concentration (Million/ml)
Old	Mean \pm SD	120 \pm 29.3	161 \pm 90.6	19.0 \pm 10.9	85.6 \pm 11.3	21.4 \pm 13.6	45.5 \pm 8.32	93.6 \pm 5.25	71.9 \pm 18	3.43 \pm 4.99	10.6 \pm 7.27	69.8 \pm 13.5	3.82 \pm 3.45	1.66 \pm 1.81
	Min-Max	80-210	23.8-634	3.57-58.6	33.4-100	0.200-64	19.2-64.3	66.6-99.2	16.3-97.4	0.915-49	2.32-44	18-92	0-19	0-9.02
Young	Mean SD	131 \pm 35.4	192 \pm 104	24.8 \pm 15.8	87.0 \pm 9.08	22.0 \pm 14.2	46.4 \pm 6 8.98	93.3 \pm 5.89	75.5 \pm 15.2	4.66 \pm 8.31	11.2 \pm 67.27	67.3 \pm 15.9	4.37 \pm 5.35	2.15 \pm 2.44
	Min-Max	80-280	29.7-588	3.86-165	53.2-99.9	0.330-60.6	25.9-84.2	54.8-99	31.2 - 97.3	0.91 - 47.4	1.65-52.5	15.9 - 89	0 -37	0-13.5

CHAPTER 5: PRE-BUT NOT POST-MEIOTIC SENESENCE AFFECTS SPERM QUALITY AND REPRODUCTIVE SUCCESS IN THE NORTH AFRICAN HOUBARA BUSTARD

Léna Meunier^{1,2}, Gabriele Sorci², Hiba Abi Hussein¹, Yves Hingrat¹, Noé Rehspringer², Michel Saint Jalme³, Loïc Lesobre¹, Janaina Torres Carreira^{1*}

¹ Reneco International Wildlife Consultants LLC, Abu Dhabi, United Arab Emirates

² Biogéosciences, UMR 6282 CNRS, Université de Bourgogne Franche-Comté, Dijon, France

³ Centre d'Ecologie et des Sciences de la Conservation, UMR 7204 MNHN CNRS-UPMC, Museum National d'Histoire Naturelle, Paris, France

⁴ Emirates Center for Wildlife Propagation, Missour, Morocco

*** Correspondence:**

Corresponding Author

jtcarreira@reneco.org

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Abstract

Age-dependent reduction in reproductive success can arise due to multiple factors including a deterioration of reproductive physiology. Senescing males have been shown to produce ejaculates with poor sperm quality, which impinges on male reproductive success. In addition to individual age, gamete age can also affect male reproductive success. Accordingly, variance in male reproductive success can be due to pre-meiotic (referring to individual age) and post-meiotic senescence (sperm age). Here, we tested whether male senescence and sperm cell aging have additive or interactive effects on male reproductive success in a bird with a promiscuous mating system, the North African Houbara Bustard. To assess the effect of pre-meiotic aging, we compared male reproductive success between two age classes (3-6- and 12–16-year-old). To infer the effect of post-meiotic aging, male ejaculates were collected at three-time intervals following a common initial collection (day 1, 5, and 10). Therefore, day 1 ejaculates are supposed to contain younger sperm than day 5 and 10 ejaculates. Following controlled artificial inseminations, reproductive success was assessed using three fitness-linked traits (hatching success, chick growth rate and survival). In addition to reproductive output, we also assessed whether pre- and post-meiotic aging affected a wide range of sperm and ejaculate traits. In agreement with previous reports, we found that males in the older age class produced less sperm



with poorer motility compared to young individuals. However, contrary to the prediction, we found that ejaculates collected at day 5 and 10 tended to have better sperm traits such as motility and velocity. The results on sperm traits were generally mirrored in the effect on reproductive success since young males produced offspring that grew faster and had better survival during the first month of life, and eggs fertilized by sperm collected at day 5 had the highest hatching success. In any of the models, there was evidence for interactive effects of male and sperm age. Overall, these results confirm the role of pre-meiotic aging on male reproductive success. The lack of evidence for sperm aging could come from the experimental design but might also reflect the pattern of mating frequency in a species with a lek-based mating system.

KEYWORDS aging, chick survival, reproductive success, sperm age, sperm quality



Introduction

Senescence is the progressive decline of physiological functions with age, resulting in increased mortality risk (actuarial senescence) and decreased reproductive output (reproductive senescence) (Monaghan et al., 2008). While evidence in supporting senescence to be a widespread phenomenon in nature has been accumulating during the last decade (Nussey et al., 2013), the study of male reproductive senescence has been restricted to a narrower range of species (Lemaître and Gaillard, 2017). Despite this limited taxonomic spectrum, current evidence in humans and captive species of mammals, fish, birds and insects suggests that male age can have profound effects on several traits defining ejaculate and sperm quality (Gasparini et al., 2010; Johnson et al., 2015; Carreira et al., 2017; Herrera-Cruz et al., 2018; Colasante et al., 2019; Fricke and Koppik, 2019; Farooq et al., 2020). For instance, old males have been reported to produce lower ejaculate volume with impaired antioxidant defenses and containing sperm with reduced motility and velocity (Wolf et al., 2000; Sartorius and Nieschlag, 2010; Noguera et al., 2012; Gunes et al., 2016).

In addition to these phenotypic changes, old males are also likely to accumulate mutations in the germline due to the continuous cell divisions that are inherent to spermatogenesis (Kong et al., 2012; Thomas et al., 2018). Finally, epigenetic changes of the germline were shown to occur during aging (Immler, 2018; Evans et al., 2019). The combined effect of phenotypic and (epi)genetic changes of sperm due to male age can therefore compromise reproductive success, both by reducing the probability to fertilize the ovum and increasing the risk of embryo/offspring mortality (Hale et al., 2008; Kim et al., 2011; Fay et al., 2016; Lippens et al., 2017; Ruhmann et al., 2018).

Besides these pre-meiotic (organismal) effects of aging, cellular senescence (post-meiotic senescence) can also contribute to determine male reproductive success. Sperm cells are particularly vulnerable to the environmental conditions they are exposed to, and susceptible to oxidative damage (Reinhardt, 2007). Sperm cells are prone to oxidative damage because they (i) produce high amount of reactive oxygen species (ROS) due to their high number of mitochondria and intense metabolic activity, (ii) have limited anti-oxidant defenses due to their reduced cytosol, (iii) do not have known mechanisms of DNA repair, and (iv) have membranes that are rich in unsaturated fatty acids which increase ROS production via oxidative carbonyls (Aitken, 2020).

After their production during spermatogenesis, spermatozoa are stored in male ducts (epididymis and vas deferens) and after mating they can also be stored in the female reproductive tract in birds (Blesbois and Brillard, 2007). During this period, both in male and female sperm storage, oxidative damage was shown to compromise sperm motility, viability, and DNA integrity in studied species (reviewed by Tarin, 2000); thus, sperm aging can alter the capacity to fertilize the ovum or



impair offspring health as shown by White et al. (2008) in kittiwakes (*Rissa trydactyla*). For instance, prolonged sperm storage in male reproductive tract was also associated with decreased velocity and impaired fertilization in the Guppy (*Poecilia reticulata*) (Gasparini et al., 2014, 2017), reduced viability and increased DNA fragmentation in zebrafishes (*Danio rerio*) (Cattelan and Gasparini, 2021) and decreased fertilization, embryo development, hatching and chick condition in kittiwakes (*Rissa trydactyla*) (White et al., 2008). Similarly, sperm aging within the female reproductive tract has been shown to have negative effects on fertility, embryonic development, and hatching rate success in poultry (Nalbandov and Card, 1943; Lodge et al., 1971).

While available evidence convincingly supports the role of both pre- and post-meiotic senescence as important drivers of male reproductive success, a question that has received little attention is whether they have additive or interactive effects on reproductive output (Pizzari et al., 2008). There are several reasons to expect that pre-meiotic senescence might amplify the effect of sperm age. For instance, as the antioxidant defense system decreases with age, sperm produced by old males might be more vulnerable to oxidative damage during the storage period. In agreement with this hypothesis, sperm of old rats are more vulnerable to both *in vivo* and *in vitro* oxidative challenge than sperm from young rats (Zubkova and Robaire, 2006).

Such interactive effects of pre- and post-meiotic senescence might have important consequences on male reproductive success, and particularly so in species experiencing strong post-copulatory sexual selection (Birkhead and Pizzari, 2002). Indeed, when females mate with multiple males during the same reproductive bout, sperm compete for ovum fertilization. In this case, when old individuals compete with young males, they might fail to fertilize the ovum because of a faster rate of sperm senescence.

The aim of the present study is to investigate whether pre and post-meiotic senescence have additive or interactive effects on male reproductive success of the North African Houbara Bustard (*Chlamydotis undulata undulata*). An interactive effect between pre- and post-meiotic senescence would indicate that any effect of sperm age on sperm quality and/or reproductive success depends on male age. Previous work conducted on this species has shown that male age negatively affects ejaculate quality, hatching success and offspring growth, survival and sperm production (Preston et al., 2011, 2015; Vuarin et al., 2021). Old males also suffer from reduced fertilization success during competitive interactions with young males, even when males of both age classes provide equal number of sperm of similar motility to the insemination (Vuarin et al., 2019a). In addition to this, female houbara bustards can store sperm for weeks, as shown by females being able to lay fertile eggs



weeks after being inseminated, and approximately 70% of chicks are sired by the last male used for insemination (L. Lesobre, personal observation).

Thus, the houbara bustard appears to be a good candidate to explore the contribution of pre- and post-meiotic senescence on male reproductive success. To this purpose, we artificially inseminated female houbara bustards with sperm collected from old and young males. For each age class, we also collected sperm at different intervals with respect to a common initial sperm collection. A longer interval between the initial collection and the collection used to inseminate the female therefore indicates prolonged sperm storage and therefore older sperm. This experimental design allowed us to assess the effect of male and sperm age on several sperm traits (viability, motility, velocity, morphology, and ROS production) and reproductive success (hatching success, chick growth rate and survival). If pre- and post-meiotic senescence have synergistic effects, we predicted statistically significant interactions between male and sperm age on sperm quality and reproductive output.

Material and methods

General procedure and experimental groups

All birds used in this study are part of the Emirates Center for Wildlife Propagation (ECWP), a conservation breeding program located in eastern Morocco, aiming at reinforcing natural populations of the North African Houbara Bustard (Lacroix, 2003). This program entirely relies on artificial inseminations (Saint Jalme et al., 1994). Males and females on this experiment were born in captivity, are part of ECWP breeders flock and have been used routinely for semen collection and artificial inseminations in the previous seasons. Briefly, semen was routinely collected using a dummy female presented to males. Once the male mounted the dummy and started copulating, a glass dish was used to collect the ejaculate by pressing it gently on the male cloaca during ejaculation. The semen was immediately transferred to a 2 mL cryotube, maintained at room temperature. Ejaculates were directly assessed for motility (using a mass motility index ranging between 0 and 5, see Vuarin et al., 2019a for details), volume and sperm concentration, and diluted (1:1) in Lake 7.1 (Lake and Ravie, 1984). For the present experiment, a 20 μ l aliquot was diluted in 180 μ l DPBS-BSA (1.5%) and set aside for flow cytometry analyses (see below), while the remaining was used to inseminate females. We used semen from 307 males belonging to two age classes: 154 young males (mean \pm SD = 4.14 \pm 1.09, minmax = 3–6 years), and 153 old males (mean \pm SD = 13.58 \pm 1.61, min-max = 12–16 years). Age categories were chosen based on previous work and senescing patterns (Preston et al., 2011; Vuarin et al., 2019a). These birds were subsequently allocated to three groups (evenly with respect to male age), corresponding to three intervals of sperm collections. Specifically, an initial semen collection was



performed (day 0) and then for each group the collections were repeated after 1, 5, or 10 days. All birds experienced the 3 collection intervals, with a rest period of 12 days between cycles (Supplementary Data and Supplementary Figure 1).

Semen analysis

Flow cytometry analysis was performed using a Guava easycyte 5HT (IMV, L'Aigle, France) flow cytometer equipped with a 150-mW blue argon laser (488 nm) and 3 color photomultipliers green (525/30 nm), yellow (583/26 nm) and red (695/50 nm). For each sample 10,000 events were counted in the sperm population gate. Data were analysed using the IMV Easycyte plus software (IMV, L'Aigle, France).

Viability

Sperm viability (% live out of 10,000 cells) was assessed using Easy Kit viability (IMV, L'Aigle, France), based on propidium iodine (PI) and SYBR-14 dyes. PI enters broken plasma membrane and binds to nucleic acids provoking the emission of 617 nm red fluorescence (Gillan et al., 2005). SYBR14 dye is membrane permeable and stains only living sperm emitting 520 nm green fluorescence when binding to nucleic acids (Garner et al., 1994). Kit's 96 wells plate were loaded with 180 μ L of PBS/1.5% BSA and 20 μ L of diluted semen (1:10 in PBS/1.5% BSA). Negative control was performed diluting the sample in distilled water instead of PBS to promote membrane damage. Samples were analysed after 10 min of incubation in the dark at room temperature.

Oxidative stress

Intracellular sperm oxidative stress was measured in live cells using a combination of Dihydroethidium (DHE, 2 mM in dimethyl sulfoxide (DMSO), Sigma-Aldrich, St Louis, USA), which emits 606 nm orange fluorescence when reacting with superoxide anions O_2^- , a ROS (Zhao et al., 2005), and SYBR-14 (Sigma-Aldrich) to measure percentage of living and oxidized sperm cells (out of 10,000 cells). In a 96 well plate 180 μ L of PBS/1.5% BSA, 20 μ L of diluted semen (1:10 in PBS/BSA), 1 μ L of SYBR-14 (Sigma-Aldrich, St Louis, USA, 1 mM stock solution, 20 μ M in DMSO work solution) and 2 μ L of DHE (2 mM in DMSO) were added. Negative control was performed incubating sperm with hydrogen peroxide for 15 min at 40°C prior to analysis. Samples were analysed after 15 min of incubation in the dark, at room temperature.

Motility

In addition to the initial mass motility index, sperm motility was further evaluated with Sperm Class Analyzer software (SCA, Microptics, Barcelona, Spain). Four microliters of diluted semen (1:10 in PBS/BSA 1.5%) were placed on a Makler chamber under a negative phase contrast microscope (Olympus BX41, 10 \times objective) linked to the computer by a camera. A maximum of five fields were



analysed to reach 350 sperm cells, 25 frames on each field were evaluated. Percentage of motile sperm and velocity [curvilinear velocity VCL ($\mu\text{m/s}$)] were recorded. VCL was selected among other parameters as it refers to the velocity of the sperm real trajectory.

Morphology

To evaluate sperm morphology, semen was fixed by adding 10 μL of 4% buffered paraformaldehyde to 20 μL of semen diluted in PBS/BSA 1.5% (1:10). Then, 6 μL of this solution were placed on a slide with a coverslip (wet mount) and observed under a phase contrast microscope (Olympus BX41, objective $\times 100$, oil immersion). For each sample 100 sperm cells were classified according to 8 categories (Bloom, 1973; Alkan et al., 2002): acrosome loss, abnormal head, swollen head, midpiece defects, tail defects, cytoplasmic droplet, teratogenic sperm (double head or tail) and normal.

Artificial insemination, egg laying, and chick rearing

A total of 310 captive bred females were inseminated only with one of either 95 young males (N = 177 females, mean female age \pm SD = 10 ± 2.4 , min-max = 4–13 years) or with 74 old males (N = 133 females, mean female age \pm SD = 10.48 ± 2.45 , min-max = 3–13 years), from the three semen collection intervals. Females were inseminated with doses containing on average 26.10 million sperms (± 16.22), an average inseminated volume of 86.18 μL (± 30.29), and a mass motility index > 3.5 . The experimental inseminations were performed between the first and the second clutch with no previous insemination during the season, to guarantee the storage tubules were empty and no further insemination were performed before the experiment related eggs were collected. For each inseminated female, we aimed at collecting the first four eggs laid corresponding approximately to 2 clutches. The eggs were collected after a single homospermatic insemination. However, not all females laid 4 eggs. Overall, 599 eggs were laid, and more specifically 44 females laid one egg, 246 laid 2 eggs, 17, 3 eggs and 3 laid 4 eggs. Upon laying, eggs were collected and artificially incubated. 54 eggs showed defects and hence were not incubated, resulting in a total of 545 incubated eggs. Eggs were candled at day 8 to check embryo development. When no signs of fertility were detected, they were opened to discriminate early embryonic death from non-fertilized eggs. The percent of successfully hatched eggs (hatching success) was recorded. Body mass of hatchlings was measured daily (to the nearest of 1 g) from day 2 to 60 post-hatching. Any mortality occurring during this period was also recorded. Chicks were reared and manually fed in cages until 35 days and after transferred to outdoor aviaries.

Statistical analyses

Statistical analyses were performed using R 3.6.0. To investigate the effect of male and sperm age (approximated by the day of ejaculate collection) on sperm quality, we ran linear mixed-effect



models [nlme package, (Pinheiro et al., 2021)] or generalized linear mixed-effect models [glmmTMB package, (Brooks et al., 2017)]. These models included male age, the day of collection and their interaction as fixed effects. Moreover, to account for seasonal variation in sperm quality, the date of each ejaculate collection (designated hereafter as “date”), was also included in the models as a polynomial term of degree three since visual inspection showed a non-linear relationship with the response variables. Male identity was included as a random effect to account for dependency between samples collected from the same male at different time of the season. The effect of male and sperm age on hatching success was assessed using a generalized linear mixed-effect model. For offspring survival, we restricted our analysis to the first 35 days of life since, according to the breeding protocol, chicks are transferred to different breeding facilities and conditions might interfere or mask the genuine effects of father age and sperm age. However, for reference, analyses up to 60 days are reported in Supplementary Figure 2 and Supplementary Tables 4, 5). Chicks’ survival was modeled using the mixed effects Cox proportional hazards models (coxme package, Therneau, 2012). Offspring growth rate up to day 60 was modeled using a linear mixed-effect model. Preston et al. (2015) showed an effect of mother age on hatching success, therefore we decided to include it in these models as a quadratic fixed effect term in addition to male and sperm age, and hatching date (to correct for possible variation across the season). For hatching success and survival models, female and male identities were used as crossed intercept random effects to account for dependency between siblings, while the female and individual identities were used as nested intercept random effects in the offspring growth rate model to account for dependencies between siblings and repeated measures of the same chick. Finally, the model on offspring growth rate also included offspring age (and squared age), offspring sex, and the interactions between male and offspring age as additional fixed effects, to account for any potential difference in growth rate between sexes and father age groups. Model families were chosen based on the type of response variable and its distribution. Stepwise backward elimination approach based on the corrected Akaike Information Criterion (Mazerolle, 2006) was used to select the best parsimonious model (the model with the lowest AICc) (Arnold, 2010; Aho et al., 2017). The model assumptions regarding the normality of residuals, homoscedasticity, and independency were checked. In case of assumption violation, data transformation, within-group variability and/or autocorrelation modeling were performed. The marginal effects plots based on the models were generated using the ggeffects R package (Lüdtke, 2018). Differences in sample size across models are due to missing values. More details about the model building and selection are given in Supplementary material.



Ethical statement

The breeding program runs under the approval of the Moroccan Ministry of Agriculture and a mandate independent veterinarian (mandate number: 534–98), providing control of the wellbeing of the birds. Onsite veterinary facilities ensure the best possible care for sick or injured birds by a team of expert veterinarians, and standards from sanitary authorities are regularly controlled.

Results

Ejaculate attributes and sperm traits

Both male and sperm age affected volume and sperm number in the ejaculate. Young males produced ejaculates with larger volume and higher number of sperm (Table 1 and Figures 1A,B). Ejaculates collected after a longer interval (day 5 and 10) from the common collection also had larger volume and more sperm (Table 1 and Figures 1A,B), suggesting that the sperm population kept accumulating with time. The interaction between male and sperm age (day of collection) was not significant and was not retained in the selected most parsimonious model, neither for ejaculate volume nor for number of sperm in the ejaculate. Young males also had a higher proportion of motile sperm in the ejaculate compared to old males (Table 1 and Figure 1C), and higher sperm curvilinear velocity (VCL) (Table 1 and Figure 1D). As for ejaculate volume and number of sperm, both proportion of motile sperm and sperm velocity were higher in ejaculates collected at day 5 and 10 (Table 1 and Figures 1C,D). Male age was not retained in the most parsimonious selected models for the percentage of sperm with normal morphology, the percentage of dead sperm, and the percentage of DHE positive sperm per ejaculate (Table 2 and Figure 2). However, the percentage of sperm with normal morphology increased as the collection interval increased (Table 2). Similarly, the percentage of dead sperm per ejaculate was lower when ejaculates were collected after the longest interval from the initial collection (Table 2 and Figure 2A). However, the percentage of DHE positive sperm (a measure of oxidative stress) increased with day of collection, suggesting higher oxidative stress in sperm with prolonged storage time (Table 2 and Figure 2B).

Reproductive success

Over the 545 eggs that were incubated, 393 hatched (72.11%). Hatching success did not differ according to male age (however, older females laid eggs with lower hatching success) (Table 3 and Figure 3A). Females inseminated with samples collected at day 5 had the highest hatching success (Table 3 and Figure 3A). The interaction between male and sperm age was not significant and not retained in the most parsimonious model. The delay between insemination and egg laying was similar for females inseminated with ejaculates collected at the 3 different intervals (Supplementary Data and Supplementary Table 2; Day 1 mean \pm SD = 6.97 \pm 2.23 days, Day 5 mean \pm SD = 7.88 \pm 2.17 days, Day



10 mean \pm SD = 7.62 \pm 2.01 days). Offspring sired by old males had a slower growth rate during the first 60 days compared to offspring sired by young males, as shown by a significant interaction between male and offspring age (Table 3). Offspring sired by young males gained on average 0.5 g per day more than those sired by old males, which resulted in a weight difference of 27 g at 60 days [mean weight of chicks sired by old males = 736.37 g (SE = 6.60, CI:723.44, 749.30) for females and 915.68 g (SE = 5.75, CI: 900.43, 930.23) for males; mean weight of chicks sired by young males = 763.62 g (SE = 7.61, CI:752.05, 774.59) and 942.60 g (SE = 6.84, CI:928.89, 955.67) for females and males, respectively]. Neither sperm age alone, nor the interaction between father and sperm age were significant predictors of offspring growth rate.

Twenty-one chicks out of 393 hatchlings died during the first 35 days of life. Offspring sired by young males had a better survival rate compared to offspring sired by old males (Table 3 and Figure 3B). A higher mortality rate was mainly seen in the first 3 days of chicks' life. Neither sperm age, nor the interaction between male and sperm age affected offspring survival.

Discussion

The aim of the present study was to investigate preand post-meiotic senescence and their potential interaction in males of a bird species with a promiscuous mating system, the houbara bustard. In agreement with previous work conducted on the species, we found that pre-meiotic effects are important determinants of ejaculate and sperm quality, and male reproductive success. However, contrary to our prediction, we found that prolonged sperm storage did not impair sperm quality and did not reduce reproductive success, in both young and old males. Thus, we did not find any evidence suggesting that male age might accelerate post-meiotic senescence. The deleterious effect of male age on sperm quality and reproductive success was expected as it has already been reported in the houbara bustard and other species. Ejaculate attributes such as ejaculate volume, sperm number and mass motility index have been previously shown to increase during the first years of life up to the age of 5–6 years and then gradually decline (Preston et al., 2011).

Here, we confirmed these previous findings since old males (mean age of 13.58 years old) produced ejaculates with lower volume and with less sperm. We extended previous findings by using flow cytometry and computer assisted techniques to assess several sperm traits that collectively contribute to define sperm quality. We found that young males had a higher percentage of motile sperm and sperm with higher velocity (VCL) compared to old individuals. However, we did not find evidence suggesting that male age affected other traits related to sperm quality such as the percentage of dead sperm or the percentage of DHE positive sperm in the ejaculate corresponding to the intercellular ROS production, a marker for oxidative stress. These results suggest age-specific effects



on different components of sperm and ejaculate quality, similar to the observation that different phenotypic traits have different rates of age-dependent decline (e.g., Lecomte et al., 2010; Hayward et al., 2015).

In addition, to reducing ejaculate and sperm quality, father age has also been shown to affect negatively both offspring growth (Preston et al., 2015) and post-release offspring survival (Vuarin et al., 2019a) in the houbara bustard. Here, we also found that offspring sired by old males had a slightly slower growth rate during the first 60 days of life and a higher mortality rate during the first 35 days of life in captivity, indicating high fitness cost due to reproductive senescence in this species. Despite the statistical significance of the difference in growth rate between paternal age groups, its biological relevance and impact on fitness should be further investigated, especially in the wild.

Contrary to our prediction, post-meiotic aging did not impair sperm quality and reproductive success. We used a proxy of post-meiotic aging, since we compared ejaculates that were collected at different intervals with respect to a common initial collection: the longer the interval, the longer the storage period, and we assumed that a longer storage would increase the proportion of old sperm in the ejaculate. Similar reasoning was used in other studies to assess the effect of sperm age on reproductive success. For instance, White et al. (2008) equipped male kittiwakes with rings on the cloaca preventing sperm transfer during mating. Some birds wore the rings for few days, others up to 2 weeks. Once the rings were removed, birds were able to copulate again, and the longer the period of ring wearing the older the sperm transferred. Using this elegant experimental design, they found that sperm aging indeed impaired male reproductive success, in terms of hatching success and hatchling condition (White et al., 2008). In a more recent study, sperm of male zebrafishes were initially collected, then males were isolated during 4, 7, or 12 days (sexual rest), after which their sperm were collected again, imposing sexual rest and consequently sperm storage in males, resulting in reduced sperm velocity (Cattelan and Gasparini, 2021).

However, it should be fully acknowledged that other studies have failed to report the predicted effect of sperm age on sperm quality. For instance, Firman et al. (2015) did not find any evidence of decreased sperm quality in house mice (*Mus domesticus*) experiencing a 2-month sexual rest compared to sexually active males. Even within species, the effect of sperm age might be inconsistent since, contrary to Cattelan and Gasparini (2021), a previous work conducted also on zebrafishes found that old (stored) sperm had higher velocity compared to freshly produced ones (Vega-Trejo et al., 2019). Despite an experimental design similar to those adopted in previous studies, we did not find evidence for deleterious effects of sperm aging. Ejaculates collected at day 5 and 10 had higher volume and contained more sperm compared to ejaculates collected at day 1, showing that sperm



accumulated in the epididymis and vas deferens over time, in the absence of mating opportunities. However, unexpectedly, the increase in sperm age resulted in a higher proportion of motile sperm in the ejaculate, higher velocity, higher proportion of sperm with normal morphology, and less dead sperm in the ejaculate. The only phenotypic trait potentially suggesting a possible decline in sperm quality with storage was the higher proportion of DHE positive sperm. However, this did not produce any deleterious effect in terms of male reproductive success since eggs fertilized with sperm after a prolonged storage had a higher hatching success and sperm age did not have any effect on chick growth rate and survival.

There are several possible explanations for these results. It is possible that our experimental design failed to produce a substantial change in the age distribution of sperm. The effect of sexual abstinence has been extensively studied in humans (Ayad et al., 2018), and recently, Okada et al. (2020) found an increase on DHE positive cells in male ejaculates with a 4-day abstinence in comparison to 1 day. They suggested that this increase would appear when sperm surpass the physiological necessary epididymal transit. The distribution of sperm age at a given time accumulated in male tract will depend on a differentiation/death process, where new sperm are produced, age and die. In the absence of sperm release (during sexual rest), the variance in sperm age will increase, with the occurrence of old and new sperm in the subsequent ejaculate. This could explain an increase in volume and sperm number along with some level of substitution of dead by viable sperm cells with time, partially explaining why the percentage of dead sperm and defective cells is improved with storage in our study.

Physiological loss of sperm cells in the male tract to the cloaca and by duct cells absorption in birds is expected at some rate, however, in domestic fowls it needed more than 4 weeks of storage to observe dead sperm cells being processed by the duct structure (Tingari and Lake, 1972). Nothing is known about such process in bustards, if it happens and at which rate. With this respect, it should also be noted that the longest rest duration in our experimental design corresponds to the average duration of spermatogenesis in birds [ca 10 days in Japanese quails (*Coturnix japonica*) 12 days in barbary drakes, 13–15 days in domestic fowls (Jones and Lin, 1993)]. How sperm are stored in the male reproductive tract can have profound consequences on the reproductive success of males (Reinhardt, 2007). Moreover, if the number of sperm released in the ejaculate is large enough, the reproductive success might still be ensured by the young sperm present in the ejaculate. This also highlights the possible role of post-copulatory selection (including cryptic female choice) as a filter promoting younger sperm within the ejaculate to fertilize the ovum.



Finally, it could be that sperm aging occurring post-mating in the female reproductive tract might obscure any effect of prolonged storage in the male reproductive tract. Particularly, this can occur if the rate of sperm aging follows a non-linear (accelerating) trend. To explore this issue, we compared the delay between insemination and egg laying (a proxy of the time sperm spent in the female reproductive tract) and found no difference between the experimental groups (the delay between insemination and egg laying being around 7 days in the three sperm age groups, see Supplementary material). This suggests that any additional effect of sperm aging in the female reproductive tract was constant across experimental groups.

According to the literature sperm stays quiescent inside the females STT, losing motility and only being released for fertilization of the ovulated oocyte (Sasanami et al., 2013). Molecular exchanges between sperm membrane and STT/female tract fluids do occur, and lactic acid concentrations for example play an important role on sperm release and retention (Matsuzaki et al., 2015). Moreover, molecules of the utero vaginal junction involved in sperm storage have been identified and have shown to improve sperm viability *in vitro* when added to the diluent (Matsuzaki et al., 2020) but no evidence of sperm remodeling or cell maturation *per se* were described so far. However, once more, little is known about avian species and especially wild models.

Alternative explanations for our findings refer to the process of post-testicular sperm maturation. In mammals, sperm released in the lumen of the seminiferous tubules at the end of spermatogenesis are not able to fertilize the ovum, yet. During their transit through the epididymis, sperm undergo several morphological and biochemical changes such as nuclear compaction, modification of the membrane composition, loss of cytoplasmic droplet and gain of factors necessary for oocyte binding, leading to sperm capacitation (James et al., 2020). However, in birds, the role of sperm maturation in the epididymis remains unclear. The epididymis is much shorter in birds compared to mammals and sperm transits through the epididymis faster due to the high rate of sperm production. Indeed, spermatogenesis is a much longer process in mammals compare to birds as it ranges from 35 days in the mice to 74 days in man (Bennett, 1977). Furthermore, artificial insemination with testicular sperm does produce fertile eggs in poultry which suggests that maturation in the epididymis is not a necessary condition for ovum fertilization (Munro, 1938; Howarth, 1983). However, other work has suggested that maturational changes do occur in birds as well. For instance, increasing sperm motility and velocity through epididymal transit has been reported in the Domestic Fowl (Munro, 1938; Ahammad et al., 2011a) and in the Japanese quail (Clulow and Jones, 1982). Similarly, in poultry, viability and fertilization capacity gradually increase from testes to vas deferentia (Ahammad et al., 2011a,b).



The ejaculate is not only made of sperm cells. Indeed, after ejaculation spermatozoa are transported by seminal plasma, a very complex fluid composed of a diversity of molecules. Therefore, sperm cells interact with seminal proteins and other molecules impacting sperm physiology and fertilization outcome (Druart and de Graaf, 2018). Proteomics studies of seminal plasma in avian species have identified proteins associated with fertility in the Turkey (Słowinska et al., 2017), in the Chicken (Labas et al., 2015) and also with age and sperm velocity in the Red Jungle Fowl (Borziak et al., 2016). More recently, extracellular vesicles were found in chicken seminal plasma with different size and proteins markers between fertile and sub fertile males (Cordeiro et al., 2021). Another promising topic is the differences in miRNAs in high- and low-quality semen samples, miRNA patterns can be different depending on the male status and environmental changes, and differences between high and low motility samples were recently described in roosters (Xing et al., 2022). Further work comparing the composition of seminal plasma and the presence of different patterns of miRNAs of ejaculates between different storage time and between old and young males in houbara bustards would be of great interest.

As for organismal aging, the rate of sperm aging is certainly not constant neither among-species nor among-individuals within species. The rate of sperm aging can be modulated by several factors (see Reinhardt, 2007 for a review). However, investment into mechanisms that limit age-associated damage does not come without cost. Therefore, natural selection could have shaped the evolution of anti-aging mechanisms according to potential fitness costs of accumulating old sperm in the ejaculate. In several bird species, females mate several times with the same male. Repeated copulations with the same male might provide freshly produced sperm for ovum fertilization. Actually, in some social species, females have been observed to eject sperm when mating occurs too early with respect to egg laying (White et al., 2008), which might represent a female strategy to avoid fertilization with old sperm. Spontaneous sperm discharge has also been suggested in males of several bird species, as shown by the presence of sperm in the cloaca of individuals sampled during the spring migration (before the mating season had started) (Quay, 1985).

Sperm release might be a mechanism allowing males to get rid of sperm that had been stored for long time before reaching the breeding ground. The North African Houbara Bustard is a solitary bird dwelling at very low density in rocky deserts and steppes (Monnier-Corbel et al., 2022). Although males cluster in leks during the mating season, the lek sites are dispersed over much larger areas compared to classical lekking species. Therefore, the mating frequency of males is certainly very low and probably most of them only mate a handful of times (or maybe never) during the whole mating period. In species where males have reduced sexual opportunities, it is straightforward to predict the evolution of mechanisms allowing to protect stored sperm from oxidative attack.



This might also apply to sperm that are stored in the female sperm storage tubules which preserve the sperm viability for weeks. Our experimental set up involved homospermic (single male) inseminations. Accordingly, our measure of reproductive success only refers to the male capacity to fertilize eggs that hatch and produce viable offspring. Obviously, a different picture might emerge when ejaculates of different males compete for ovum fertilization. Female houbara bustards do mate with multiple males during the same reproductive bout, therefore there is scope for sperm competition to operate in this species (Lesobre et al., 2010). Under sperm competition, selection on traits promoting ovum fertilization is exacerbated (Vuarin et al., 2019b). For instance, small reduction in motility might have no consequences under homospermic mating, whereas sperm with lower motility can be easily outcompeted if females mate with multiple males. Previous work conducted on the houbara bustard has shown that when sperm of young and old males compete for ovum fertilization (during mixed sperm inseminations), young males have a substantial advantage since they have a larger paternity share compared to the 1:1 null hypothesis (Vuarin et al., 2019a). Therefore, an extension of the present work could consider performing competitive inseminations using sperm of different ages. Future work should also extend the period of sperm storage to fully assess the possible duration of sperm lifespan and perhaps increase the likelihood to observe sperm aging in this species.

Data availability statement

The original contributions presented in this study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

Ethical review and approval was not required for the animal study because the breeding program runs under the approval of the Moroccan Ministry of Agriculture and a mandate Independent Veterinarian (mandate number: 534–98), providing control of the wellbeing of the birds. Onsite veterinary facilities ensure the best possible care for sick or injured birds by a team of expert veterinarians, and standards from sanitary authorities are regularly controlled.

Author contributions

YH, MS-J, LL, GS, and JT conceived the study. LM, NR, and JT collected the data. HAH analysed the data and prepared the tables and figures. YH and LL curated and supervised the conservation breeding program. LM and GS wrote the first draft of the manuscript. All authors edited, revised, and approved the final version.



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Tables and figures

Table 1: Selected parsimonious linear mixed models exploring the effect of male and sperm age on the ejaculate volume, the number of sperm in the ejaculate, the percentage of motile sperm (on the logit scale), and the curvilinear velocity (VCL). In total, 667 sperm samples were analysed. We report the parameter estimate with the 95% confidence intervals, t- and p-values. See text and online supplementary material for details on the initial model structure and model selection.

Response variable	Fixed effect	Estimate	95% CI	t-value	p-value
Ejaculate volume N= 665	Intercept	8.33	7.93, 8.73	40.69	<0.001
	Male age (young)	0.63	0.14, 1.12	2.53	0.011
	Collection day (5)	0.48	0.12, 0.84	2.63	0.009
	Collection day (10)	0.36	0.02, 0.69	2.08	0.038
	Date	-5.22	-9.13, -1.31	-2.61	0.009
	Date ²	-5.56	-9.33, -1.79	-2.88	0.004
Number of sperm in the ejaculate N= 593	Intercept	3.22	3.08, 3.37	43.50	<0.001
	Male age (young)	0.42	0.27, 0.58	5.32	<0.001
	Collection day (5)	0.48	0.36, 0.60	7.78	<0.001
	Collection day (10)	0.74	0.63, 0.85	13.05	<0.001
	Date	1.28	0.06, 2.49	2.05	0.040
	Date ²	-2.66	-3.80, -1.53	-4.59	<0.001
	Date ³	-1.04	-2.21, 0.11	-1.78	0.0757
Motile sperm (%) N= 658	Intercept	1.15	0.95, 1.35	11.15	<0.001
	Male age (young)	0.24	0.01, 0.46	2.03	0.0434
	Collection day (5)	0.61	0.39, 0.82	5.50	<0.001
	Collection day (10)	0.56	0.36, 0.76	5.41	<0.001



	Date	-5.45	-7.73, -3.16	-4.66	<0.001
	Date²	0.59	-1.63, 2.81	0.52	0.604
	Date³	-3.44	-5.74, -1.15	-2.93	0.003
VCL N=658	Intercept	31.76	30.46, 33.07	47.52	<0.001
	Male age (young)	1.80	0.39, 3.20	2.51	0.012
	Collection day (5)	2.80	1.27, 4.30	3.61	<0.001
	Collection day (10)	2.56	1.13, 3.98	3.51	<0.001
	Date	-36.38	-52.20, -20.56	-4.50	<0.001
	Date²	49.07	33.62, 64.53	6.21	<0.001
	Date³	-60.73	-76.56, -44.89	-7.50	<0.001

N= Number of observations. CI: Confidence Intervals. Significance level: p -value \leq 0.05

Table 2: Selected parsimonious linear mixed models exploring the effect of male and sperm age on the percentage of normal, dead, and DHE positive sperm. We report the parameter estimate with the 95% confidence intervals, t - and p -values. See text and online supplementary material for details on the initial model structure and model selection.

Response variable	Fixed effect	Estimate	95% CI	t-value	p-value
Normal sperm (%) N= 650	Intercept	0.52	0.41, 0.63	9.13	<0.001
	Collection day (5)	0.33	0.21, 0.45	5.22	<0.001
	Collection day (10)	0.43	0.31, 0.55	7.20	<0.001
	Date	-2.11	-3.44, -0.77	-3.10	0.002
	Date²	-3.05	-4.34, -1.76	-4.65	<0.001
Dead sperm (%) N= 666	Intercept	2.07	1.98, 2.17	42.37	<0.001
	Collection day (5)	-0.05	-0.17, 0.07	-0.74	0.460



	Collection day (10)	-0.25	-0.36, -0.13	-4.28	<0.001
	Date	1.43	0.12, 2.73	2.14	0.033
	Date²	2.81	1.55, 4.07	4.37	<0.001
	Date³	-1.85	-3.16, -0.54	-2.78	0.006
DHE (%) N= 667	Intercept	1.05	0.88, 1.22	12.04	<0.001
	Collection day (5)	0.20	-0.01, 0.41	1.83	0.068
	Collection day (10)	0.45	0.25, 0.65	4.43	<0.001
	Date	-7.71	-10.00, -5.42	-6.60	<0.001
	Date²	0.90	-1.30, 3.11	0.80	0.422
	Date³	-2.25	-4.55, 0.05	-1.92	0.056

N= Number of observations. CI: Confidence Intervals. Significance level: p -value \leq 0.05

Table 3: Selected parsimonious generalised mixed linear models exploring the effect of male and sperm age on hatching success, offspring survival and growth rate. We report the parameter estimate with the 95% confidence intervals, t - and p -values. See text and online supplementary material for details on the initial model structure and model selection. The chicks for which the sex remained unknown were excluded from the data set of the offspring growth rate model.

Response variable	Fixed effect	Estimate	95% CI	t-value	p-value
Hatching success N= 545	Intercept	-0.97	-2.71, 0.77	-1.09	0.276
	Collection day (5)	0.88	0.95, 1.46	2.95	0.003
	Collection day (10)	0.45	-0.05, 0.96	1.76	0.078
	Date	0.03	0.01, 0.04	3.17	0.001
	Mother age	-0.08	-0.17, 0.01	-1.73	0.084

Offspring survival (HR) N= 393	Father age (young)	0.27	0.10, 0.72	-2.60	0.009
	Date	568.26	0.37, 8.66*e ⁵	1.70	0.090
	Date²	13041.1 3	19.29, 8.82*e ⁶	2.85	0.004
Offspring growth rate N= 379	Intercept	199.54	201.76, 197.92	175.95	<0.001
	Father age (young)	3.35	0.98, 5.72	2.79	0.0058
	Offspring sex (female)	-19.78	-22.07, -17.49	-17.09	<0.001
	Offspring age	16523.0 3	16289.47, 16756.59	138.61	<0.001
	Offspring age²	2621.11	2507.88, 2734.32	45.36	<0.001
	Offspring age: father age (young)	466.67	230.56, 702.77	3.87	0.0001
	Offspring age²: father age (young)	139.71	24.55, 254.88	2.38	0.0175
	Offspring age: offspring sex (female)	-2907.43	-3146.17, -2668.70	-23.86	<0.001
	Offspring age²: offspring sex (female)	-1107.05	-1222.64, -991,47	-18.77	<0.001

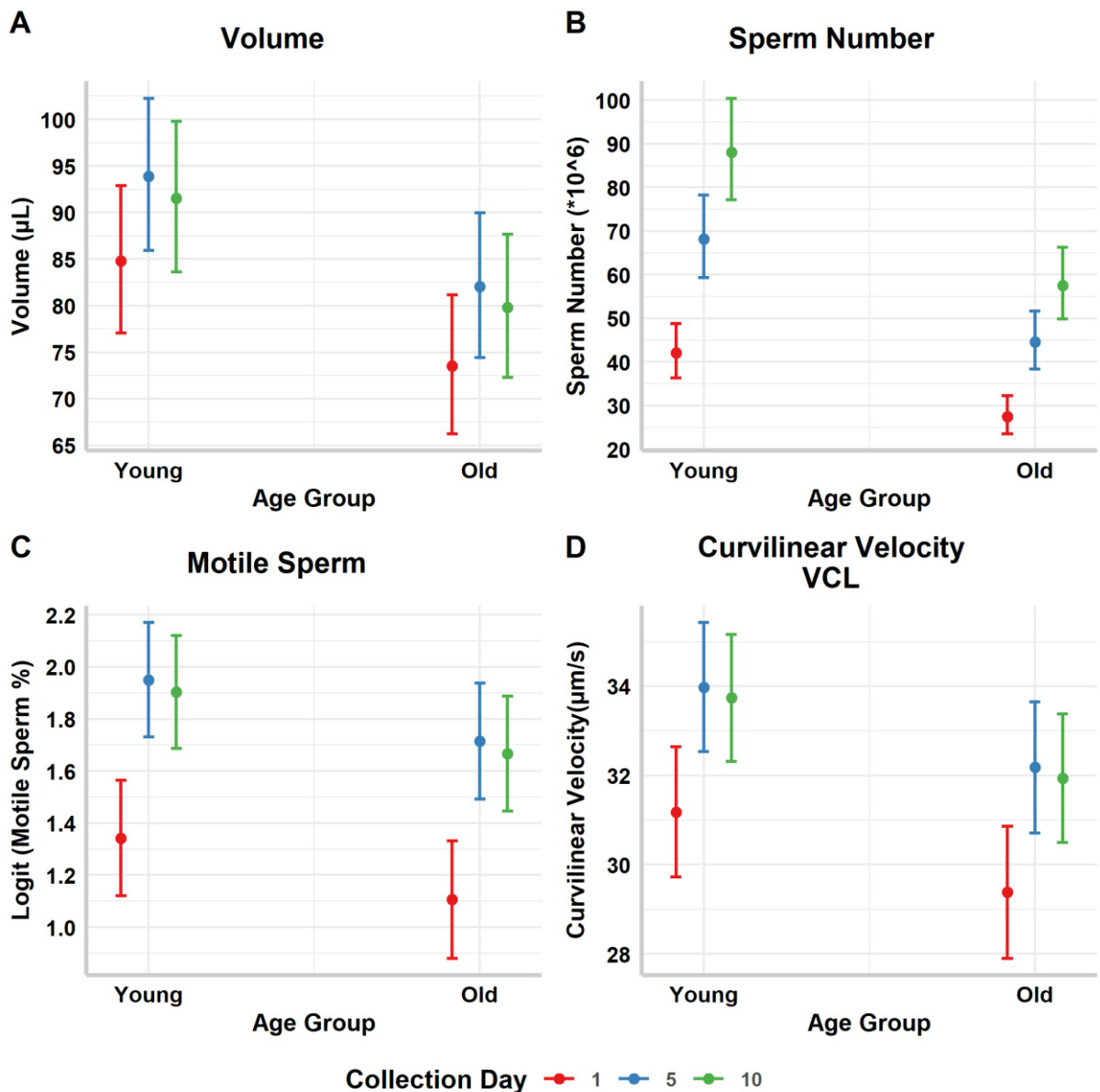


Figure 1: Predicted marginal means and their corresponding 95% confidence intervals of the (A) ejaculate volume (μl), (B) number of sperm in the ejaculate (x 10⁶), (C) percentage of motile sperm (on the logit scale), and (D) curvilinear velocity, per male (old vs. young) and sperm age. Collection day refers to the day sperm was collected after a common initial collection, and it is used as a proxy of sperm age.

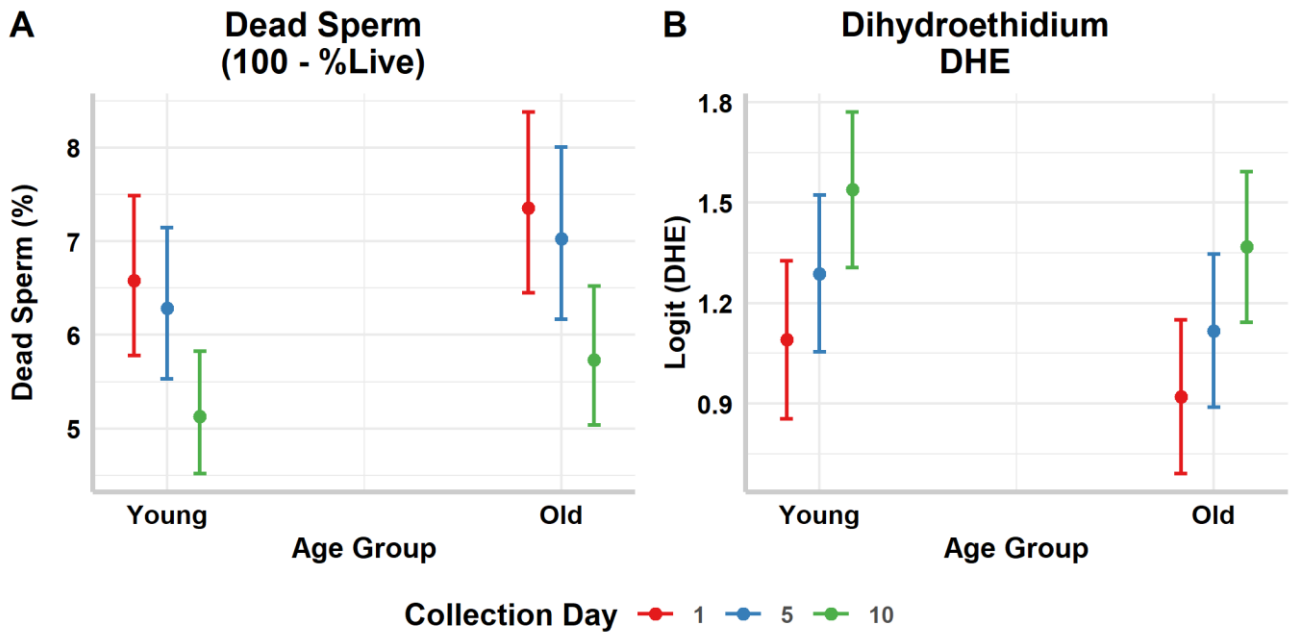


Figure 2: Predicted marginal means and their corresponding 95% confidence intervals of the (A) percentage of dead sperm, (B) and the percentage of DHE positive sperm (on the logit scale), per male (old vs. young) and sperm age. Collection day refers to the day sperm was collected after a common initial collection, and it is used as a proxy of sperm age.

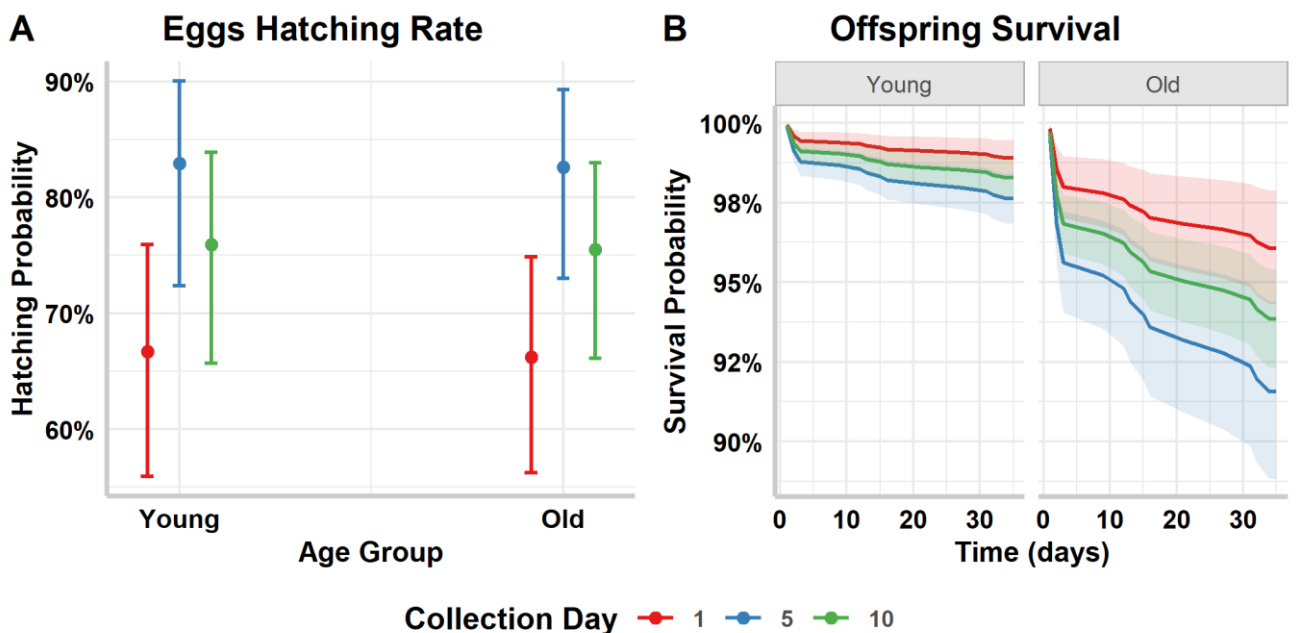
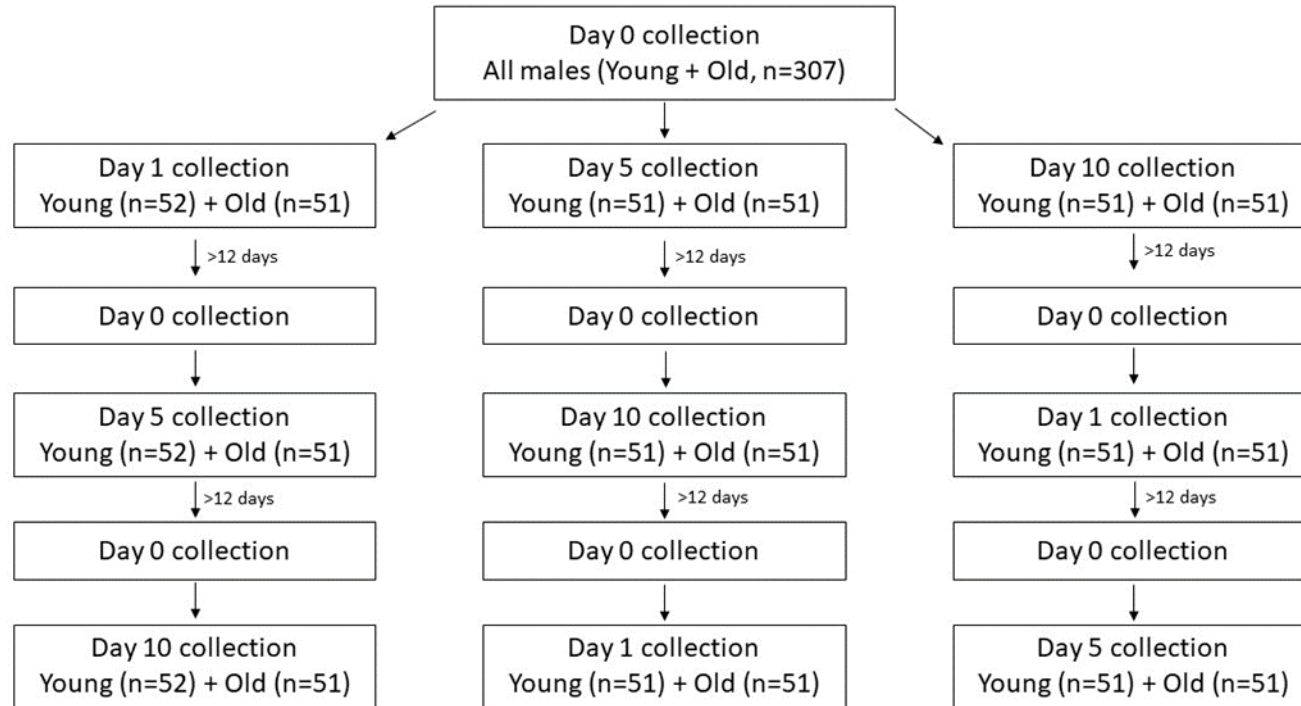


Figure 3: Predicted marginal means of the (A) hatching success and the corresponding 95% confidence intervals, (B) and chick survival during the first month of life and the corresponding standard errors, per male and sperm age. Collection day refers to the day sperm was collected after a common initial collection, and it is used as a proxy of sperm age.

Supplementary Material

Experimental design



Supplementary Figure 5. Experimental design and distribution of groups. 154 young males (3-6 years), and 153 old males (12-16 years were distributed equally in three groups evenly with respect to male age), corresponding to three intervals of sperm collections, 1, 5 and 10 days after an initial collection (day 0). All birds were submitted to the 3 collection intervals with a minimum rest period of 12 days between cycles.



Model building and selection procedure

We ran linear mixed models and generalized linear mixed models to investigate the effect of male age (two classes: young vs. old) and sperm age (approximated by the day of semen collection following a common initial collection: day -1, and then day 1, 5, and 10) on traits describing sperm quality and reproductive success. Model selection procedure was based on a stepwise backward approach. Model families were chosen based on the distribution of the response variable. We started with the saturated model in which all the covariates, interactions and random effects were included. At this stage, based on diagnostic plots, residuals variance homogeneity and autocorrelation were diagnosed; in case of violation(s), variance and/or autocorrelation was/were respectively modelled through the inclusion of the appropriate dispersion terms (Brooks et al, 2017), variance and/or autocorrelation function(s) (Pinheiro et al, 2015). Then, one term was removed at a time; only terms having a significant contribution to the outcome were returned in the model. The significance of each model term was assessed based on the corrected Akaike's Information Criterion (**AICc**), the top model corresponding to the most parsimonious model with the lowest AIC (Arnold, 2010 and Aho et al., 2017).

The following tables report for each response variable the terms included in the initial model, the error distribution (**Family**), variance and/or autocorrelation term(s) inclusion or random effects exclusion (**Other Terms**), degrees of freedom (**df**), Bayesian Information Criterion (**BIC**), the corrected Akaike's Information Criterion (**AICc**), the difference in AIC units compared to the top model (**Δ AICc**), and the AICc relative weights (**Weight**).

All the statistical analyses were performed using R 3.6.0.; namely *nlme* package for the linear mixed models (Pinheiro et al., 2021), *glmmTMB* package for the generalized linear mixed models (Brooks et al., 2017), and the *coxme* and *survival* packages for the survival model (Therneau, 2012; Therneau 2022), the *AICcmodavg* (Mazerolle, 2006) for the computation of AICc and AICc weight.

Ejaculate and sperm quality models

To test for the effect of male and sperm (day of collection) age on ejaculate and sperm quality, male age, collection day and their interaction were included as fixed effects. In addition to these terms, the day of semen collection (plus squared and cubic terms) were also included to correct for seasonal variation in sperm quality. The male identity was used as intercept random effect.

Supplementary Table 1: Model building and selection for ejaculate and sperm quality traits.

Response variable	Male age	Collection day	Male age: Collection day	Date	Date ²	Date ₃	Family	Other terms	df	BIC	AICc	ΔAICc	Weight
Ejaculate volume Square root transformation	*	*		*	*		Gaussian		8	3029.98	2994.20	0.00	0.71
	*	*		*	*	*			9	3036.47	2996.25	2.05	0.25
	*	*	*	*	*	*			11	3049.21	3000.12	5.92	0.043
Number of sperm in the ejaculate	*	*		*	*	*	Negative Binomial		9	5879.01	5839.85	0.00	0.62
	*	*	*	*	*	*		11	5888.63	5840.85	1.00	0.38	
	*	*	*	*	*	*	Poisson		10	9080.82	9037.34	3197.49	0.00
Motile sperm (%) Logit transformation	*	*		*	*	*	Gaussian		9	2189.98	2149.86	0.00	0.80
	*	*	*	*	*	*			11	2201.66	2152.69	2.83	0.20
VCL	*	*		*	*	*	Gaussian		9	4690.88	4650.76	0.00	0.87
	*	*	*	*	*	*			11	4703.55	4654.58	3.82	0.13
Dead sperm (%) Logarithmic transformation		*		*	*	*	Gaussian		8	1486.95	1450.67	0.00	0.47
	*	*		*	*	*			9	1490.91	1451.15	0.48	0.37
	*	*	*	*	*	*			11	1501.90	1452.79	2.12	0.16

DHE positive sperm (%) Logit transformation		*		*	*	*	Gaussian		9	2265.72	2225.47	0.00	0.46
	*	*		*	*	*			10	2270.43	2225.73	0.26	0.40
	*	*	*	*	*	*		+ VarIdent (~Age Group) ¹	12	2281.39	2228.05	2.58	0.13
	*	*	*	*	*	*			11	2281.61	2232.26	6.79	0.02
Normal Sperm (%) Logarithmic transformation		*		*	*		Gaussian		8	1587.38	1551.79	0.00	0.65
		*		*	*	*			9	1593.72	1553.71	1.92	0.25
	*	*		*	*	*			10	1600.19	1555.76	3.97	0.09
	*	*	*	*	*	*		+ VarIdent (~Age Group) ¹	12	1612.78	1559.55	7.75	0.01
	*	*	*	*	*	*			11	1613.66	1564.82	13.03	0.00

¹VarIdent models heteroscedasticity and allows the variance to be different for each level of the age group factor. (Pinheiro and Bates,2000).

Delay between insemination and egg laying

In addition to aging due to prolonged sperm storage in the vasa deferentia, sperm keep aging in the female reproductive tract, until egg fertilization. To check whether sperm aging in the female reproductive tract might bias the results based on sperm storage in the male reproductive tract, we compared the delay between insemination and egg laying for the different experimental groups and found that for all groups the delay was homogenous and around 7 days.

Supplementary Table 2: Mean and standard deviation of the delay between insemination and egg laying, according to male age and semen collection day. N stands for the number of observations within each group.

	Old N= 230	Young N= 315	Collection Day 1 N= 208	Collection Day 5 N= 144	Collection Day 10 N= 193
Mean	7.43	7.45	6.97	7.88	7.62
SD	2.16	2.18	2.23	2.17	2.01

Hatching rate

Following previous results (Preston et al, 2015), the initial model on hatching rate, in addition to father age, day of collection and their interaction, also included mother age (and squared mother age). Seasonal variation was modelled including hatching date (plus squared and cubic date). The mother and father identities were included as intercept crossed random effects to account for the dependencies between siblings.

Supplementary Table 3: Hatching rate stepwise backward model selection procedure.

Response variable	Father age	Collection day	Male age: Collection day	Date	Date ²	Date ³	Mother age	Mother Age ²	Family	Other terms	df	BIC	AICc	ΔAICc	Weight
Hatching Rate		*		*			*		Binomial		6	656.91	631.27	0.00	0.62
		*		*			*	*			7	663.18	633.29	2.02	0.23
	*	*		*			*	*			8	669.48	635.34	4.07	0.08
	*	*		*	*		*	*			9	674.63	636.26	5.00	0.05
	*	*		*	*	*	*	*			10	680.94	638.34	7.07	0.02
	*	*	*	*	*	*	*	*		-(1 FatherID) ₂	12	693.49	642.47	11.20	0.00
	*	*	*	*	*	*	*	*			13	699.79	644.57	13.30	0.00

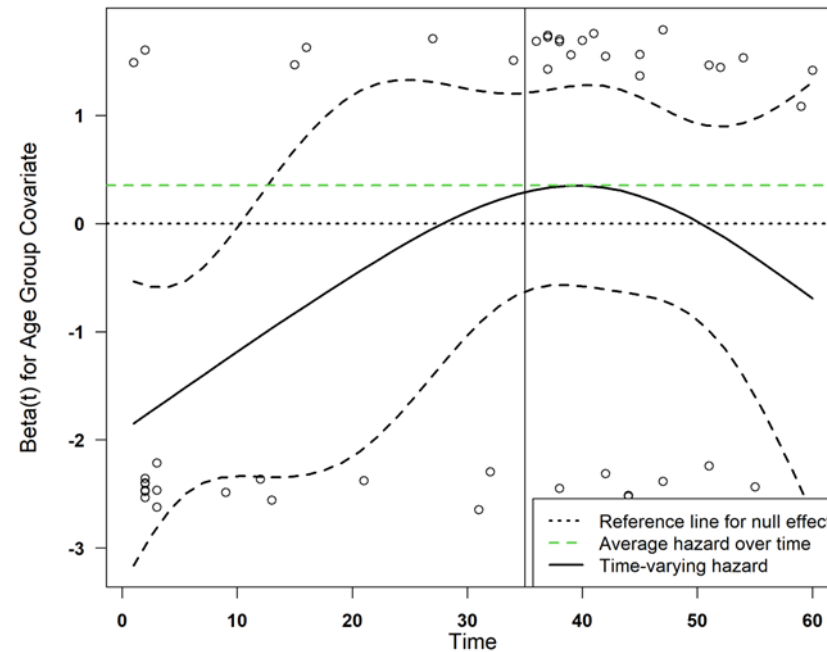
²-(1|FatherID): exclusion of the random effect term since its standard deviation was close to zero.

Offspring survival Offspring survival was modelled using mixed proportional hazard models. The first model was built on the chicks' survival data that goes up to the 60 first days of life. The proportional hazards' assumption was assessed based on scaled Schoenfeld residuals and showed an assumption violation specifically on the effect of father age group which showed a non-constant relative hazard over time with a changing point accruing around 35 days of life (Figure S.1, Table S.4).

Supplementary Table 4: Test of the proportional hazards' assumption for the Cox regression model on data between day 1 and day 60 of life. A significant deviation from the proportional hazards' assumption was detected for the age group covariate.

	Chi-square	df	<i>p value</i>
Collection day	0.033	2	0.984
Age group	5.084	1	0.024
Collection day: Age group	2.105	3	0.551
Date	1.855	2	0.395
Schoenfeld's global test	9.947	8	0.269

Based on figure S.2, we have identified around 35 days a turning point at which the effect of age group reached the average hazard over time; hence, as recommended by Therneau and Grambsch (2000), a time-varying covariate model was fit by splitting the time of the analysis into 2 intervals ([0,35] and]35,60]) and considering the effect of age group on survival in each time interval. The result of the time varying covariate model is given in table S.5.



Supplementary Figure 2: Plot of the estimate of the time-dependent coefficient ($\beta(t)$) for age group covariate; it shows that the effect of the covariate age group on the mortality varies over time. The confidence intervals of the covariate effect (black solid line) are showed by the upper and lower dashed black lines and the average hazard over time is given by the dashed green line.

The effect of age group is significant in the first time interval between 1 and 35 days of life (Table S.5). The turning point identified based on the model could be explained by the breeding protocol procedures according to which the chicks are transferred during the 5th week of life to a different breeding location and conditions; therefore, after 35 days different other reasons could interfere and mask the effect of age group on the chicks' mortality. The proportional hazard assumption was validated on the time-varying coefficient Cox model.

Supplementary Table 5: Selected parsimonious time-varying coefficient Cox model exploring the effect of male and sperm age on offspring survival.

Response variable	Fixed effect	Estimate	95% CI	z-value	p value
Offspring survival (HR) N= 393	Father age (young):]0,35]	0.26	0.1, 0.69	-2.60	0.006
	Father age (young):]35,60]	1.25	0.55, 2.84	0.538	0.591
	Date	5318.39	0.95, 2.97*e7	1.95	0.051
	Date ²	4039.63	16.87, 9.67*e5	2.97	0.003
	Date ³	0.012	2.62*e ⁻⁵ , 5.24	-1.43	0.153

N= Number of observations. CI: Confidence Intervals. Significance level: p-value ≤ 0.05

We report the parameter estimate with the 95% confidence intervals, z- and p-values.

Therefore, we have decided to present in the article the result of the model developed on survival data and mortalities that have occurred between day 1 and day 35 of life only. Table S.6 displays the survival model selection procedure.

Supplementary Table 6: Offspring survival proportional hazard model selection procedure.

Response variable	Father age	Collection Day	Father age: Collection Day	Date	Date ²	Date ₃	Mother age	Mother age ²	Family	Other terms	k	BIC	AICc	ΔAICc	Weight	
Offspring survival	*			*	*				Cox Model		4	254.54	238.75	0.00	0.46	
	*			*	*	*					5	259.71	239.99	1.25	0.25	
	*			*	*	*	*				7	265.46	241.01	2.26	0.15	
	*			*	*	*	*	*			6	268.53	241.83	3.09	0.10	
	*	*		*	*	*	*	*			9	279.85	244.56	5.81	0.03	
	*	*	*	*	*	*	*	*			- (1 FatherID) ²	11	289.79	246.77	8.02	0.01
	*	*	*	*	*	*	*	*				12	295.76	248.90	10.15	0.00

²-(1|FatherID): exclusion of the random effect term since its standard deviation was close to zero.

Offspring growth rate

Body mass (g) of offspring was measured daily starting from the age of 2 and until the age of 60 days. To model growth rate (change in body mass with age), the model included, in addition to the fixed effects described in the tables above, offspring age (and the squared term), the interactions between the father age and offspring age, as well as the interaction between offspring sex and offspring age. The mother and offspring identities were included as nested intercept random effects to simultaneously account for dependencies between siblings having the same mother and repeated measures on the same chick. Father identity was not included in this model since the crossed random effects' structure is not yet supported by nlme package. However, the previous results of hatching rate and offspring survival showed no statistical support for the inclusion of the father identity as random effect; hence, its exclusion should not affect the results.

Supplementary Table 7: Offspring body mass stepwise backward model selection. The polynomial terms were grouped together due to the large number of variables included in this model, and they were designated by (Age + Age²) for offspring age, the hatching date by (Date + Date² + Date³), and the mother age by (Mother Age + Mother Age²).

Response variable	Father age	Collection day	Father age: Collection day	Sex	Age + Age ²	(Age+ Age ²): Father age	(Age+ Age ²): Sex	Date +Date ² +Date ³	Mother age+ Mother age ²	Family	Additional Terms	df	BIC	AICc	ΔAICc	weight	
Offspring body mass	*			*	*	*	*			Gaussian		14	79317.55	79217.63	0.00	0.80	
	*	*		*	*	*	*					16	79335.02	79220.82	3.20	0.16	
	*	*		*	*	*	*	*				19	79359.57	79223.98	6.36	0.03	
	*	*	*	*	*	*	*	*	*				21	79377.02	79227.17	9.54	0.01
	*	*	*	*	*	*	*	*	*		*	corCAR1() ³	23	79395.27	79231.16	13.53	0.00
	*	*	*	*	*	*	*	*	*		*	varPower() ⁴	22	81388.83	81231.84	2014.22	0.00
	*	*	*	*	*	*	*	*	*		*		21	90762.76	90612.91	11395.28	0.00

³ corCAR1(): continuous-time autoregressive correlation structure of order 1 to model the residuals autocorrelation (Box et al., 1994; Jones et al., 1993; Pinheiro and Bates, 2000).

⁴ VarPower: power law function used to model the heteroscedasticity. The variance is modelled as a power of the absolute value of a covariate, which in our case corresponds to the fitted values (Pinheiro and Bates, 2000).

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CHAPTER 6: DISENTANGLING THE IMPORTANCE OF EJACULATE ATTRIBUTES, AGE AND MATING ORDER IN A SPECIES WITH A LAST MALE PRECEDENCE EFFECT

Abstract

Sperm competition can exert strong selection pressure on ejaculate and sperm traits that are functionally related to the fertilization of the egg. However, the outcome of the male-male competition also depends on other features potentially unrelated to sperm quality. This includes mechanisms of female control over-fertilization and mating order. Here, we used an experimental approach to disentangle the importance of sperm traits, male age, and mating order during competitive fertilizations in the North African Houbara Bustard, a bird species with a lek-based mating system. To this purpose, we artificially inseminated females twice with sperm collected from males at prime and senescing age, with 48 hours between the two inseminations. We also characterized sperm quality of competing males using different metrics. We found that sperm used for inseminations occurring 48 hours after the first insemination had a higher fertilization success, irrespective of male age and sperm quality. Therefore, this result shows that mating order plays a key role during competitive fertilization and confirms that the last male precedence operates in this species. We then explored to what extent the difference in the quality of competing sperm could offset the last male precedence. We found that ejaculates used for first inseminations did outcompete ejaculates used for last inseminations only when they were of higher quality in terms of key traits such as motility, velocity or normal morphology. We did not find evidence supporting the hypothesis that sperm from males at prime age outcompeted sperm from senescent males. Although these results stress the importance of mating order and sperm traits as drivers of male reproductive success, they also show that males of relatively inferior quality might still succeed in fertilizing the eggs if they can manage to secure the last position in the mating sequence.

Introduction

Sexual selection refers to the process of differential reproductive success of individuals, that, in species with internal fertilization, depends on two sequential stages (Andersson 1994). The first step involves accessing a mate, and the second step accessing the gametes. Since failure to succeed in any of these two steps results in nil reproductive success, it is straightforward to understand that there is strong selection pressure to maximize the expression of traits playing a role in the pre-and post-copulatory stages (Simmons et al. 2017). However, the intensity of the selection also depends on the specific ecological characteristics of the species, being the strongest, for instance, in species with promiscuous mating systems or with biased operational sex ratios (Emlen and Oring 1977).

In particular, when females mate with multiple males during the same reproductive bout, the sperm of different individuals will compete to fertilize the eggs (Birkhead and Moller 1998). Sperm competition is a widespread phenomenon occurring in both vertebrates and invertebrates (Birkhead and Pizzari 2002). As mentioned above, sperm competition should exert strong selection pressure on traits whose function is related to fertilization success (Simmons and Fitzpatrick 2012; Fitzpatrick and Lüpold 2014). In agreement with this hypothesis, several studies have reported evidence showing that in species where males are at high risk of sperm competition, there is a substantial increase in the investment into traits supposed to improve fertilization success (ejaculate size, sperm quality) compared to species with less intense post-copulatory selection (e.g., Lüpold et al 2020). Similar results have been reported at the within-species/among-individual level (e.g., Bartlett et al. 2017).

The outcome of sperm competition is not only driven by male-specific traits. Females can also determine the fate of sperm competition in several ways (Eberhard 1996; Firman et al. 2017). In particular, in many species, females can store the sperm transferred during different matings in specific structures (e.g., sperm storage tubules in birds) (Holt and Fazeli 2016). Depending on the species, stored sperm can be used to fertilize the eggs up to several weeks/months post-mating. Female storage, therefore, potentially paves the way for a female control over fertilization success (Cardozo et al. 2020; Demont et al. 2021). Moreover, in both internal and external fertilizers, sperm are exposed to chemo-attractants contained in follicular and ovarian fluids that can also contribute to determining the relative success of competing sperm (Gasparini et al. 2020). For instance, in the Zebrafish (*Danio rerio*), an external fertilizer, traits important for fertilization success (velocity, motility, and longevity) are improved when sperm are exposed to ovarian fluids (Poli et al. 2019). Importantly, the effect of exposure to the ovarian fluids depended on male and female identity, showing that individual-specific chemo-attractants have the potential to drive the outcome of sperm competition (Poli et al. 2019). In an elegant experiment, Fitzpatrick et al. (2020) exposed human sperm to female chemo-attractants in gamete-based mate choice trials. They found that sperm were consistently attracted by specific



follicular fluids, supporting the idea that the interaction between ovarian fluids and competing sperm is one important mechanism underlying female cryptic choice (Gasparini et al. 2020).

Another female mechanism of control refers to the order with which different males will mate prior to egg laying (Evans and Magurran 2001; Sorci et al. 2023). In internal fertilizers, mating always occurs sequentially implying that some males will mate last in the mating sequence (close to egg laying). In this case, there is evidence showing that males in the last position of the mating sequence have a substantial advantage compared to the other males, a phenomenon called the last male precedence effect (Birkhead et al. 1995; Briskie 1996; McKone 2023; Sorci et al. 2023), although in some cases first mating males appear to have preferential access to ova (Rodrigues et al. 2020). When males mating at the last (or first) position in the mating sequence have an advantage, females might exert control on the outcome of the sperm competition simply by mating with preferred males as first or last mates. Evidence supporting the role of premating choice on the outcome of post-copulatory selection comes from an experiment where female Guppies (*Poecilia reticulata*) either were allowed to mate naturally with two males or were artificially inseminated with sperm collected from the same two males (Gasparini and Evans 2018). In agreement with the hypothesis that female control on mating drives the outcome of sperm competition, paternity share between the two competing males was much more skewed when females were naturally mated than when females were artificially inseminated (Gasparini and Evans 2018).

The mechanisms underlying the last male precedence effect have been poorly studied due to the difficulties to follow the fate of sperm transferred during successive matings within the female reproductive tract. However, several hypotheses have been put forward, including the passive loss of stored sperm with time, the displacement of stored sperm by newly transferred one, or spatial segregation of different sperm both within and among storage tubules. In the yellow dung fly (*Scatophaga stercoraria*), last males fertilize a higher share of eggs and evidence shows that this competitive advantage arises from a larger number of sperm stored in female spermathecae (Demont et al. 2021), suggesting that sperm from last males do replace previously stored sperm. Whatever the underlying mechanism(s), in addition to offering a possible control to females on the outcome of the sperm competition, the last male precedence effect could also relax the selection acting on ejaculate and sperm trait. This should be particularly true when mating order occurs at random (no female control over mating order), the rationale being that males with relatively poor ejaculates might nevertheless secure reproductive success if they are lucky enough to mate at the end of the mating sequence.

Here, we conducted an experimental study where we aimed to explore the relative importance of mating order, traits supposed to reflect the intrinsic ejaculate/sperm quality, and male age as drivers of fertilization success. To this purpose, we used a bird species with a polyandrous mating system



where males are under strong post-copulatory sexual selection, the North African Houbara Bustard (*Chlamydotis undulata undulata*). Over the last decades, the natural populations of the North African Houbara Bustard have declined over the entire geographic range due to overhunting and habitat destruction (Goriup, 1997; Le Cuziat et al., 2005). This prompted the launch of an *ex situ* conservation program in Morocco with the aim of restoring natural populations across North Africa (Lacroix 2003). The conservation program is based on a captive breeding where females are artificially inseminated with ejaculates collected from males of the breeding flock. This gives a rather unique opportunity to experimentally disentangle the relative contribution of random mating order, intrinsic ejaculate quality, and male age to fertilization success. We inseminated females with sperm collected from dyads of prime and senescing males, with 48 hours between the two inseminations. In one group, females were first inseminated with sperm collected from prime age birds and then with sperm from senescing individuals, and in the other group the order was reversed (first insemination = senescing males; second insemination = prime age). Several dimensions of the ejaculate/sperm quality space were also assessed. We used prime and senescing individuals, because previous work conducted in this species has shown that senescing males produce ejaculates containing a smaller number of sperm of lower quality (Preston et al. 2011; Meunier et al. 2022). Accordingly, using ejaculates of prime and senescing males should increase the competitive advantage of individuals with better ejaculate attributes (i.e., prime age males).

With this experimental design, we formulated the following predictions: 1) males whose sperm has been used for second inseminations should have a higher fertilization success; 2) when sperm from prime-age birds are used for second inseminations, the paternity share of last males should be more pronounced; 3) only when the within-dyad difference in ejaculate/sperm quality is substantially skewed towards males used for first inseminations, we predicted a lower paternity share of last males.

Material and Methods

Animals and general procedure

All birds used for this experiment were part of a conservation breeding programme [Emirates Center for Wildlife Propagation (ECWP)] of the North African Houbara Bustard located in Missouri, (Morocco). Birds were housed individually in outdoor pens and received specially formulated dry food pellets, mealworms, and crickets as food and water *ad libitum*.

Experimental design

Females (n = 142, aged 3 to 10 years) were allocated to two experimental groups. Females in the first group (n = 69) were artificially inseminated with sperm collected from a prime-age male (3 to 5 year old) and 48 hours after, they were inseminated again with sperm collected from a senescing



male (14 to 20 year old). Females in the second group ($n = 73$) were first inseminated with sperm collected from senescing males and then (48 hours after) with sperm from a prime age male. These were the first inseminations females received during the 2022 breeding season, which excluded any possible effect of sperm stored from previous inseminations.

A total of 284 ejaculates were collected from 144 males (64 old and 80 prime age males). Ejaculates were collected in the morning by trained ECWP staff members using a dummy female. The dummy female was presented to the male and the ejaculate collected in a Petri dish held under the male cloaca. This should ensure that the quantity of semen collected closely approaches the amount ejaculated during a natural copulation (at least compared to other semen collecting techniques, such as massage). Ejaculates were immediately transferred to a 2 mL cryotube. One aliquot of 10 μl was then used to assess the number of sperm in the ejaculate using a spectrophotometer (600nm, Jenway 6051, Cole-Parmer, USA), a second aliquot of 20 μl was used to assess sperm quality (see “Ejaculate attributes and sperm traits” section), and the remaining volume (if $> 50 \mu\text{l}$) was used for the inseminations.

Females were inseminated through the slow delivery of sperm inside the vagina using a positive displacement pipette (Saint Jalme et al., 1994). To reproduce as faithfully as possible the natural conditions, ejaculates from young and old males were not matched in terms of sperm quantity or quality. The only selection criterion that was applied concerned the volume of the ejaculate (minimum volume inseminated = 50 μl . Given that 30 μl were used for the analysis of sperm quantity and quality, ejaculates with volumes smaller than 80 μl were discarded. Females were inseminated with a mean ejaculate volume of 94.04 μl (sd = 25.67 μl , min-max = 50-130 μl , $n = 142$).

Females were allowed to lay and the eggs were collected upon the day of laying and placed in artificial incubation up to 24 days. One hundred twenty-one females (out of the initial 142) laid at least one egg. Whenever possible, two clutches per female were collected (ranging from 2 to 6 eggs). A total of 386 eggs were laid but 31 of them had cracked shells or had defects and were not incubated. Therefore, only 355 eggs were incubated. Fertility was assessed at day 8 of incubation by candling. If the eggs were not showing any signs of fertility (developed air chamber, visible embryo, or blood vessels), they were opened. Eggs that did not hatch after 24 days of incubation were also autopsied. A total of 213 eggs hatched.

Ejaculate attributes and sperm traits

To investigate whether differences in the attributes of the ejaculates used for first or last inseminations could reverse the last male precedence effect, we focused on five traits: the number of sperm inseminated, sperm motility (percentage of motile sperm in the ejaculate), curvilinear velocity



of motile sperm ($\mu\text{m}/\text{sec}$), sperm viability (percentage of living sperm in the ejaculate), and sperm morphology (percentage of sperm with normal morphology in the ejaculate).

The number of sperm inseminated was estimated based on the number of sperm contained in the 10 μL aliquot used in the spectrophotometer and the ejaculate volume used for the insemination.

Sperm motility and velocity were assessed using the Sperm Class Analyzer (SCA) software (Microptics, Barcelona, Spain). A total of 275 ejaculates of 139 males were analysed. Four microliters of diluted semen (1:20 in PBS/BSA 1.5% for most samples, 1:10 or 1:40 if the concentration was too low or too high) were placed on 20 μm depth disposable chamber (4 chambers/slide, Proiser, ISAD4C20) under a negative phase contrast microscope (Nikon Eclipse E200, 10x objective) linked to the computer by a camera. A maximum of 30 fields were analysed as to reach 500 sperm (see Table S1 in the supplementary material for details on SCA settings). We computed the percentage of motile sperm in the sample and the sperm curvilinear velocity (VCL, $\mu\text{m}/\text{s}$).

Sperm viability or membrane integrity was assessed by flow cytometry using a Guava easycyte 5HT (IMV, L'Aigle, France) flow cytometer equipped with a 150-mW blue argon laser (488 nm) and 3 colour photomultipliers green (525/30 nm), yellow (583/26 nm) and red (695/50nm). Data were analysed using the guavaSoft IMV software (IMV, L'Aigle, France). A total of 271 ejaculates of 138 males were analysed using Easy Kit™ 1 viability (IMV, L'Aigle, 131 France), which contains propidium iodide (PI) and SYBR-14 dye dried and pre-loaded in 96 rounds bottoms well plates. PI exclusively enters damaged plasma membranes and binds to nucleic acids and when excited at 488nm emits red fluorescence at 617 nm (Gillan et al. 2005). SYBR-14 dye is membrane permeable and emits green fluorescence at 518 nm when bounding to nucleic acids (Garner et al., 1994), when sperm membrane is permeable by PI the red signal overtakes the fluorescent signal of the SYBR green. Twenty microliters of diluted (1:10) sample were transferred in the kit plate with 180 μL of PBS/BSA 1.5% and mixed. Negative control was made by incubating 20 μL of samples mix with 180 μL of Triton X 1% in 1.5mL cryotubes for 15 minutes in the dark at room temperature. Triton X is a detergent that permeabilizes the membranes. Samples were washed by centrifugation for 5 minutes at 1000g and resuspended in 180 μL of PBS/BSA 1.5%. Finally, 200 μL of the treated samples were loaded to the 96 wells kit plate and mixed. Samples were analysed after 15 minutes of incubation in the dark at RT on a plate shaker (Green, 525/30 nm, and red fluorescence, 695/50 nm, 10000 gated events).

Sperm morphology was assessed for 264 ejaculates of 134 males. Fixed solutions of semen were first prepared by adding 5 μL of 4% buffered paraformaldehyde (40% PFA diluted 1:10 in PBS 1X) to 10 μL of diluted ejaculate (1:10). Then 6 μL of the fixed solution were placed on a slide, covered with a coverslip and observed under a phase contrast microscope (Nikon E200-LED, Japan; objective x100, Phase contrast, oil immersion). For each sample, 100 sperm cells were observed, and each classified in 8 categories (Bloom 1973; Alkan et al. 2002): acrosome loss, cytoplasmic droplet, swollen head, head



defect, midpiece defect, tail defect, teratogenic sperm, and normal sperm cell. Samples with very low concentrations were not assessed for sperm morphology as it was not possible to count 100 cells.

Paternity assignment

Blood and tissue samples were collected from chicks and dead embryos, respectively and conserved in absolute ethanol. A total of 252 offsprings (206 chicks and 46 embryos) were sampled. Twenty microliters of blood were collected from the brachial vein from chicks at day 1 by using heparin free capillary (Hirschmann, Germany) and transferred in 180 μL of absolute ethanol. Tissue (muscle) samples (1 mm^3) were collected from dead embryos during autopsy and transferred in 900 μL of absolute ethanol. Blood samples from all parents (females and putative fathers) were collected from the brachial vein with a 3mL syringe and a needle (23G), and 100 μL were transferred in 900 μL of absolute ethanol. All samples were stored in 1 ml cryotubes with screwcaps and were stored in the freezer (-18C) until analysis.

DNA extractions were done at the National Avian Research Center (NARC) in UAE. First, blood and tissue samples were subsampled by transferring either 10 μL of blood or a small piece of tissue into an empty 1.5ml microtube. Lysis was done by adding 200 μL of cold PBS and 10 μL of proteinase K (20mg/mL) to each tube. Tubes were vortexed and left in the incubator in a thermal mixer at 56°C for 40 minutes (up to 60 minutes for tissue samples). For the next steps, extraction was made using ID Gene™ Mag Fast Extraction Kit (IDVet, France) and a DNA extraction robot AutoPure96 (BonninTech, China). After extraction, DNA concentrations were measured with a Qubit assay kit (Thermofisher, USA) for 6 to 8 random samples per plate. If the DNA was concentrated enough to be diluted (>10 ng/ μL), 50 μL of ultra-pure water were added to the elution plate and 50 μL of this dilution were transferred in a new plate, dried with and sealed to be sent to GENOSCREEN (Lille, France) for genotyping. Eight microsatellite loci designed for the North African Houbara Bustard (Chbel et al. 2002) were genotyped: A113abis, A120, A2, A210, A21, A29, D117, D118. The detailed genotyping procedure can be found in Lesobre et al. (2010).

Paternities were assigned using CERVUS.3.0.7 based on the following simulation parameters: 10,000 cycles, 2 candidate fathers, 98.58% of fathers sampled, 96.09 % loci typed, 0.05% of genotyping error and 4 minimum loci typed. Only 6 of the 8 loci genotyped were used for the analysis because two loci, A2 and A210, gave high genotyping error rates, respectively 30 and 25%. Paternity was attributed for 218 offspring based on positive LOD score of trios (known mother, offspring and candidate father). As only 4 to 8 loci could be used to attribute paternity, we decided to rerun the genotyping with additional markers. Samples were sent again to GENOSCREEN for genotyping 24 loci A113abis, A120, A2, A210, D119, A21, A29, D110, D117, D118, A10, A18, A205, A208, Otmic26, Otmic27, Otmic38 A106,



CAM-04, CAM-07, CAM-19, TG03-002, TG04-004 and TG07-022. Analysis of these samples is not finalized at the time of writing and therefore the results based on 6 loci will be used in this chapter.

Statistical analyses

Siring success was modeled using a Generalized Linear Mixed Model (GLMM) with a binomial distribution of errors (0 = failure, 1 = success). Insemination order and age class (plus their interaction) were included as fixed terms. Male ID within dyads was declared as a random effect (i.e., some males were used in different dyads). This model informed us whether there was any deviation from the 0.5 siring success under the null hypothesis of no effect of mating order and male age. We then added to this reference model, five key variables (one variable per model) describing different aspects of ejaculate/sperm attributes as fixed effects.

Finally, to assess whether ejaculate/sperm attributes could offset the expected siring advantage of last males, we computed the difference between the values of ejaculate/sperm traits of the successful minus the values of the unsuccessful male within dyads. This reduced the number of data lines to one per dyad. We then ran a GLMM with a binomial distribution of errors where the response variable was the position of the male in the insemination sequences among the successful individuals and the difference in the within dyad ejaculate/sperm values was included as a fixed effect (one trait per model). Male ID was declared as a random effect. Given that sperm traits tend to positively covary, we also used a principal component analysis (PCA) to reduce the dimensions of the ejaculate/sperm attribute space. The first component extracted from the PCA was included as a fixed effect in a GLMM where the response variable was the position of the male in the insemination sequences among the successful individuals (and male ID included as a random effect). All analyses were performed in SAS (9.4).

Results

Males whose sperm were used for inseminations occurring in the last position sired significantly more offspring compared to first inseminations ($F_{1,432} = 8.10$, $p = 0.0046$, Figure 1a). Males in the two age classes had similar fertilization success ($F_{1,432} = 0.88$, $p = 0.3499$), and the advantage experienced by males used for last inseminations did not depend on age (insemination order x age class: $F_{1,432} = 0.00$, $p = 1$; Figure 1a). A model on a restricted dataset that only included first laid eggs, provided similar results, the last male precedence effect being even stronger (60.5% of first laid eggs fertilized by the last male; Figure 1b).

When adding ejaculate/sperm attributes in the model, we found that they all consistently predicted siring success; the higher the number of inseminated sperm, sperm motility, velocity, viability, and proportion of sperm with normal morphology, the higher the fertilization success (Table



1). Adding ejaculate attributes did not change the strength of the last male precedence which ranged from 0.57 and 0.59 across models (Table 1). As for the reference model, neither age class nor the interaction between age class and insemination order were significant predictors of fertilization success (Table 1).

We expected prime-age males to have an advantage when competing with senescing males but we did not find any evidence supporting this prediction. We, therefore, investigated if the absence of difference in siring success between age classes could come from similar sperm attributes over ages. To this purpose, we computed the differences in sperm attributes within dyads (old – young) and compared them to the null hypothesis (mean difference = 0). In agreement with our prediction, we found that, within dyads, old males had consistently less sperm in their ejaculates, and therefore less sperm from old males were used for the inseminations (mean difference in the number of inseminated sperm \pm sd = $-3.78 \times 10^6 \pm 12.21 \times 10^6$, $t = -2.84$, $p = 0.0055$, $n = 105$). However, none of the other sperm features considered here differed between competing males within dyads (motility: $t = -1.59$, $p = 0.1147$, $n = 99$; velocity: $t = -1.62$, $p = 0.1080$, $n = 99$; viability: $t = -1.44$, $p = 0.1538$, $n = 101$; normal morphology: $t = 1.17$, $p = 0.2450$; $n = 90$). We used a criterion to include ejaculates for the artificial inseminations based on a minimum threshold of 80 μ l of ejaculate volume. Therefore, it could be possible that the inclusion criterion was more selective for the old age class, leaving only the best ejaculates of the best senescing individuals for inclusion in the sample. To check this hypothesis, we first compared the mean volume of the total number of ejaculates (all the ejaculates collected between February and May 2022) that were produced by the males used for the experiment. As expected, old males produced ejaculates of smaller volume (old, mean \pm sd = $86.94 \pm 39.21 \mu$ l, $n = 1819$ ejaculates; young, mean \pm sd = $106.33 \pm 48.64 \mu$ l, $n = 3305$ ejaculates; GLMM, $F_{1,149} = 9.22$, $p = 0.0028$). Therefore, the proportion of ejaculates above the 80 μ l inclusion threshold was smaller for the old age class, suggesting indeed that our inclusion criterion was more stringent for old than for young males (45.3% of the ejaculates produced by old males being below the threshold vs. 30.4% ejaculates produced by young individuals, $\chi^2_1 = 113.34$, $p < 0.0001$, $n = 5124$ ejaculates, Figure 2a). We also investigated what was the proportion of males aged between 3 and 5 years and between 14 and 20 years within the entire ECWP breeding flock (not only the individuals used for the experiment) that between February and May 2022 produced ejaculates with mean volumes below the 80 μ l threshold. Within the entire breeding flock, young males produced ejaculates of larger volume (old, mean \pm sd = $63.30 \pm 29.36 \mu$ l, $n = 344$ males; young, mean \pm sd = $69.43 \pm 33.57 \mu$ l, $n = 656$ males; GLMM, $F_{1,998} = 8.18$, $p = 0.0043$). In this sample, again the proportion of males producing ejaculates with mean volumes above the 80 μ l inclusion threshold was smaller for the old age class (77.7% vs. 68.3%, $\chi^2_1 = 9.63$, $p = 0.0019$, $n = 1000$ males, Figure 2b). Therefore, by applying a single inclusion criterion we selected the best ejaculates of



the best males of the flock, potentially accounting for the absence of age effect on sperm quality traits and paternity share between age classes.

Although sperm from males used for last inseminations had a higher paternity share compared to the 0.5 random expectation, sperm used for first inseminations did succeed to fertilize the eggs in about 40% of cases. We therefore wished to investigate if this happened according to the differences in sperm traits within dyads of competing males, irrespective of age. To this purpose, we computed the within dyads differences for each of the five ejaculate attributes between the male that succeed to fertilize the eggs and the male that did not succeed, and used these values as predictors of the likelihood of siring the eggs when in the first or last position of the insemination sequence. As predicted, for all ejaculate attributes, the within dyad differences were negatively correlated with the probability of siring the egg when in last position (the correlation being, however, non-significant for the difference in inseminated sperm and the difference in the percentage of live sperm in the ejaculate) (Table 2, Figure 3). In other words, when competing males had similar ejaculate/sperm attributes, the last male had a higher likelihood to fertilize the egg (Figure 3); however, this advantage was progressively lost if the last male had to compete with another male who expressed better ejaculate traits (Figure 3).

We also reduced the dimensions of the ejaculate/sperm attribute space using a PCA. The PCA showed that the first principal components captured most of the variance (58%) with high and positive loadings for the five traits (Figure 4). We therefore used this first component as an explanatory variable in a GLMM where the position of the siring male (first or last) was the response variable. As expected, this first component strongly predicted the probability that sperm from the first insemination succeeded to fertilize the egg ($F_{1,178} = 9.30$, $p = 0.0026$; Figure 5).

Discussion

Using an experimental approach based on artificial inseminations of female North African houbara bustards, we showed that the last male in the insemination sequence has a better siring success when competing with males of similar ejaculate/sperm quality. The advantage of the last male is however lost when the first male in the insemination sequence has better ejaculate/sperm attributes. These results therefore confirm the importance of the last male precedence effect in this species (Sorci et al. 2023) and that the strength of selection on sperm traits is weaker for males at the last position of the mating sequence.

Since the seminal paper published by Parker (1970), much emphasis has been put on the importance of sperm competition as one of the drivers of differential reproductive success (Birkhead and Moller 1998; Fitzpatrick and Lüpold 2014). Indeed, when ejaculates compete within the female



reproductive tract, several attributes can increase the likelihood to fertilize the eggs (Macartney et al. 2024). This view has however been extended to include other aspects that do not necessarily involve ejaculate and sperm attributes (Eberhard 1996; Firman et al. 2017; Lüpold et al. 2020). Namely, the competitive advantage of last males opens the question of the possible control over mating position. In certain species, males can have a control over mating sequence by preventing females from mating (e.g., mate guarding, copulatory plugs) or by repeatedly mating with the same female (Birkhead et al. 1987; Zamudio and Sinervo 2000; Schneider et al. 2016). However, females might also have a straightforward way to control the mating sequence, simply by mating with preferred males when close to laying. The hypothesis of a female control over the mating sequence has been barely investigated, however, indirect evidence shows that the outcome of sperm competition differs when females do express a choice compared to when they are artificially inseminated (e.g., Gasparini and Evans 2018). Therefore, female behaviour does appear to contribute to the process of post-copulatory selection. The third scenario implies that neither males nor females control the mating sequence, which might then depend on the random encounters between sexes. In this case, a weaker selection is expected to operate on sperm traits because relatively poor-quality males might nevertheless succeed in fertilizing the eggs if they are lucky enough to get the last position in the mating sequence. If sperm traits are heritable, which has been commonly found in several species including the North African Houbara Bustard (Chargé et al. 2013), the combined effect of last male precedence and random mating sequence might contribute to maintain the variation in traits supposed to be under strong selection. Here we provided evidence supporting this hypothesis. Sperm that were used to inseminate females at the last position fertilized about 60% of the eggs (higher than the 50% null hypothesis) and adding different metrics of sperm quality did not change the overall estimates of the last male precedence effect. This competitive advantage was maintained up to a certain threshold of difference in sperm quality between the competing males. For instance, ejaculates used at the first position in the insemination sequence did succeed in offsetting the last male precedence effect only when containing above 20% more motile sperm. Therefore, ejaculates of relatively poor quality managed to fertilize the eggs provided the penalty did not exceed 20%.

The frequency of the last male precedence effect under natural conditions is difficult to establish given that in most cases the exact number of males with which females mate and their order is unknown. Therefore, the last male precedence has almost exclusively studied in species where females can either mate under controlled conditions or are artificially inseminated (Compton et al. 1978; Carleial et al. 2020). Even harder is to identify the mechanisms underlying the competitive advantage of male depending on their position in the mating sequence. In species lacking intromitted organs, like the vast majority of birds, where males cannot physically remove sperm transferred during previous matings, the mechanisms possibly explaining the last male precedence involve the passive



loss of sperm with time, the displacement of sperm previously stored in the storage tubules by newly arriving ones, or the spatial segregation of sperm either within or across tubules (McKone 2023). In a companion study, we investigated these different possible mechanisms during competitive and non-competitive inseminations with fluorescent sperm. We only found support for the sperm displacement hypothesis since red fluorescent sperm inseminated 48 hours after a blue fluorescent sperm insemination were retrieved in higher numbers in the storage tubules, despite the number of sperm inseminated being equal (Meunier et al. submitted). Sperm displacement has been reported in some of the few species where the mechanisms of sperm competition have been studied within the female reproductive tract, including the fowl and the Fruit Fly (Manier et al. 2010; Matsuzaki et al. 2024).

We predicted a weaker last male precedence effect when last inseminations with sperm from senescing males had to compete against sperm from prime age males, but the results showed that the competitive advantage of the last male of the insemination sequence was independent of the male age class. This result might appear surprising because previous work conducted on the North African Houbara Bustard has shown that old males produce ejaculates with less sperm of poorer quality (in terms of motility, velocity and normal morphology) (Preston et al. 2011; Meunier et al. 2022). Moreover, during competitive fertilization trials using inseminations containing mixes of sperm from senescing and prime age males, the latter have been shown to have a higher paternity share (Vuarin et al. 2019). In order to investigate the reasons underlying the discrepancy between the predicted effect of male age and the observed results, we first compared the actual difference in sperm attributes between old and young males within dyads. Although we found that young males had more sperm in the ejaculate, as expected, this was the only trait that differed between age classes. Competing old and young males had indeed similar values of sperm motility, velocity, viability and normal morphology. Similar sperm quality in the sample of old and young males used for this experiment might therefore explain why paternity share did not differ between age classes. However, as mentioned above, previous work has shown that old males have poorer sperm quality compared to prime age males. A possible reason why we had individuals of similar sperm quality here might be due to the criterion that we applied to include males in the experiment. Since 50 μ l of ejaculates are needed to inseminate the females, and we needed 30 μ l for the different measurements of sperm numbers and quality, all the ejaculates smaller than 80 μ l had to be discarded. Although this was the only inclusion criterion and ejaculates were not matched up in terms of their quality, it turns out that we kept the best individuals of both age classes and particularly the best ejaculates of the best old males.

A posteriori, we can say that this inclusion criterion strongly limits the inference that can be made on the relative importance of mate order vs male age, which was the aim of the study. This is not the only limit of this work since the paternity assignment was based on a small number of microsatellite markers with quite a high genotyping error (and a high proportion of chicks for which



paternity could not be reliably assigned). Fortunately, this last limitation can be overcome since we are currently genotyping birds for a larger panel of loci. It is possible that the general results will not change, but at least we will have better data on paternity assignments both in terms of quantity (chicks whose paternity is currently undetermined) and quality (certitude of paternity assignment).

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Tables

Table 1. Generalized linear mixed models with a binomial distribution of errors exploring the association between fertilization success and five traits describing different aspects of ejaculate/sperm quality. To avoid collinearity, each ejaculate/sperm quality trait was included in a separate model. The models also included the position of males in the insemination sequence (first or last), age class (prime vs old) and the interaction between ejaculate/sperm attributes and age class. Male ID within dyads was included as a random intercept in the models to take into account the non-independence of values from the same male. We report the parameter estimate for the five ejaculate/sperm attributes with the 95% confidence intervals, and p values. We also report the Least-square means of the siring probability of the last male in the insemination sequence (last male precedence) along with the 95% confidence intervals and p values. The effect of insemination order was consistent across models with the last male siring between 57% and 59% of the offspring. Finally, we report the p values of the age class and age class x ejaculate/sperm attribute interaction and the sample size for each model. For each model, the ratio between the generalized η^2 and the df ranged between 0.99 and 1.

	Parameter estimate	95% CI	p	Last male precedence	95% CI	p	p values for age class	p values for ages class x insemination order	N males, offspring
Number of sperm inseminated	0.055	0.03/0.081	< 0.0001	0.583	0.514/0.648	0.0013	0.0941	0.7348	115.436
Sperm motility	0.038	0.016/0.06	0.0006	0.587	0.517/0.654	0.0008	0.2274	0.8128	110.413
VCL	0.044	0.021/0.067	0.0002	0.590	0.520/0.656	0.0007	0.2255	0.9412	110.413
Viability	0.078	0.028/0.127	0.0022	0.573	0.503/0.641	0.0031	0.2028	0.5033	110.409
Sperm morphology	0.031	0.016/0.045	< 0.0001	0.585	0.511/0.655	0.0017	0.6718	0.9228	106.391

Table 2. Generalized linear mixed models with a binomial distribution of errors exploring the association between position of successful males in the insemination sequence (first or last) and the within dyad difference in five traits describing different aspects of ejaculate/sperm quality. To avoid collinearity, each within dyad difference was included in a separate model. Male ID was also included as a random intercept in the models to take into account the non-independence of values from the same male. We report the parameter estimates with the 95% confidence intervals, p values, and sample size.

	Parameter estimate	95% CI	p	N males, offspring
Δ number of sperm inseminated	-0.019	-0.05/0.012	0.2196	87.218
Δ sperm motility	-0.039	-0.063/-0.015	0.0015	81.203
Δ VCL	-0.037	-0.071/-0.003	0.0341	80.201
Δ viability	-0.046	-0.102/0.01	0.1065	77.197
Δ sperm morphology	-0.03	-0.046/-0.013	0.0005	75.190

Figures

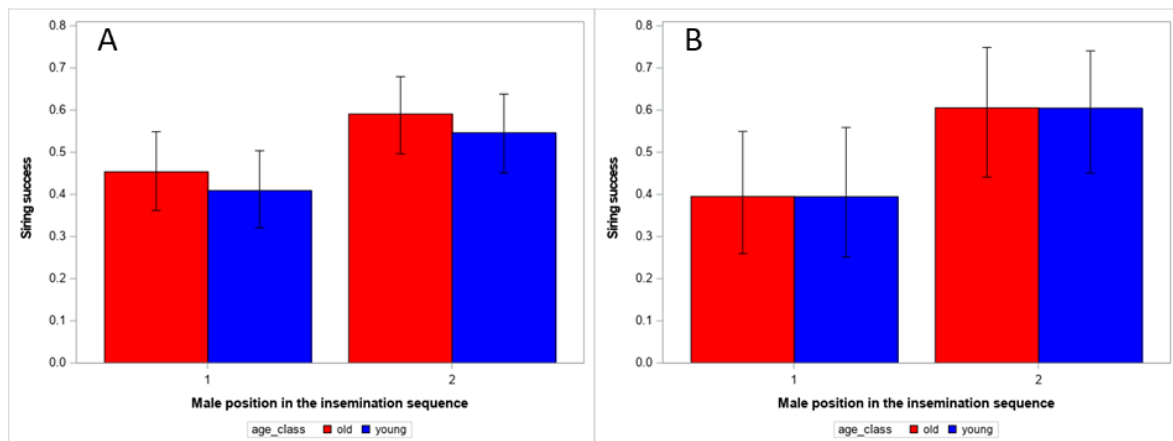


Figure 1. Siring success of male North African houbara bustards depending on their position in insemination sequence (1 = first, 2 = last) and their age (young = prime age, old = senescing). We report the Least-square means with the 95% confidence intervals. A) Siring success including all laid eggs (N = 115 males and 436 offspring); B) Siring success based only on first laid eggs (N = 100 males and 162 offspring).

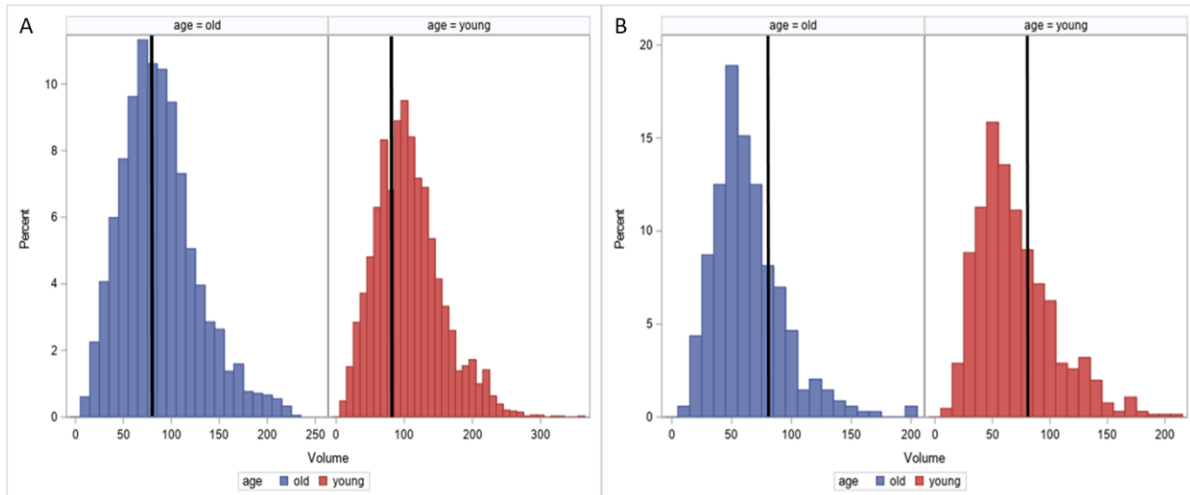


Figure 2. Distributions of ejaculate volume. **A)** Volume (μl) of total ejaculates collected from males used for the experiments over the period February – May 2022; **B)** Mean volume (μl) of ejaculates collected from males in the entire breeding flock (not only the males used of the experiment). Young and old age classes refer to 3 to 5 and 14 to 20 year old birds, respectively. The horizontal lines correspond to the 80 μl threshold for inclusion in the experiment.

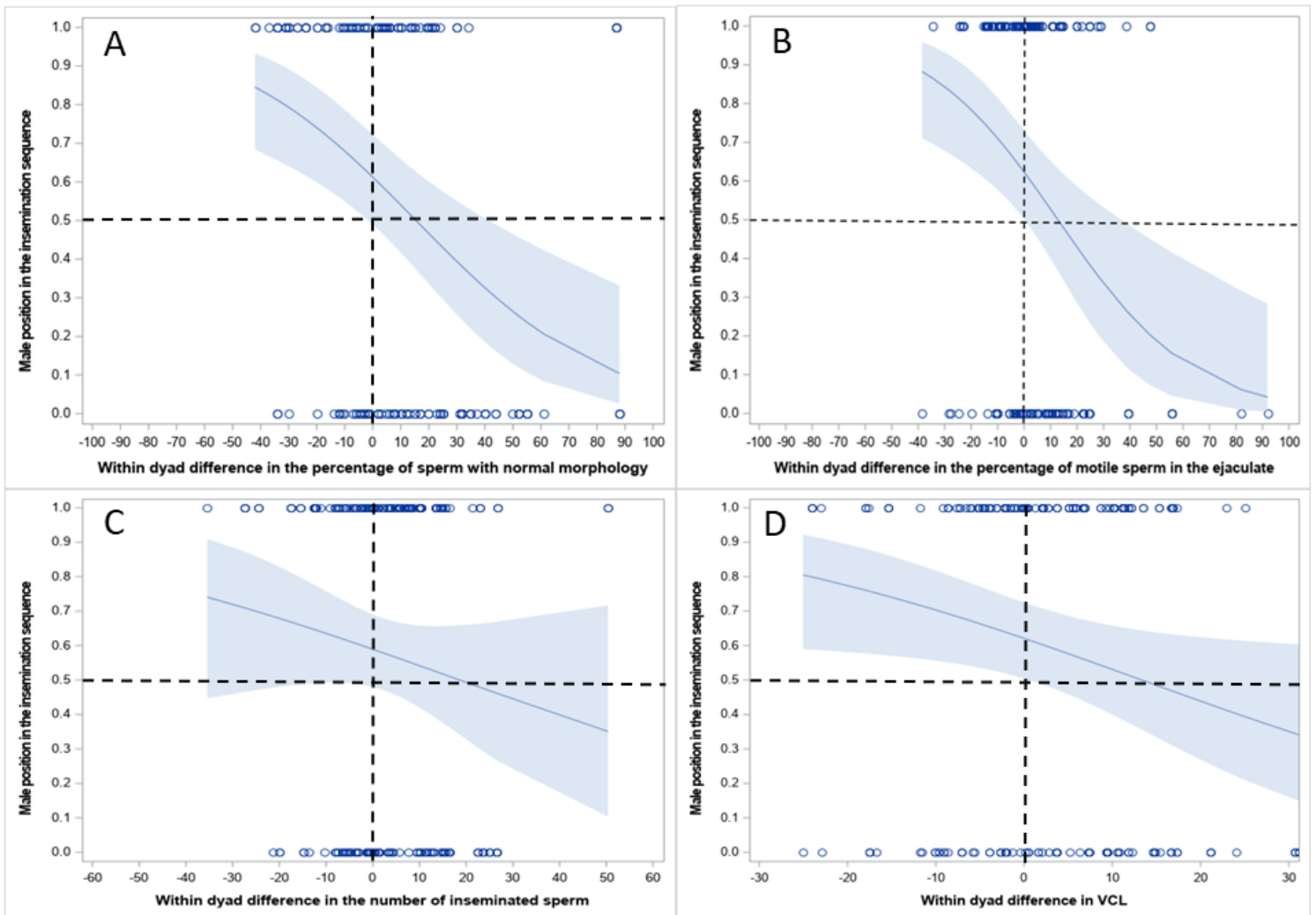


Figure 3. Relationship between the position of successful males (males that fertilized the egg) in the insemination sequence (0 = first; 1 = last) and the within dyad difference in **A)** the percentage of sperm with normal morphology in the ejaculate; **B)** the percentage of motile sperm in the ejaculate; **C)** the number of inseminated sperm; **D)** sperm curvilinear velocity ($\mu\text{m/s}$). Dotted horizontal lines represent the random expectation of 50% probability of being successful when first or last in the insemination sequence. Dotted vertical lines represent equal values of ejaculate/sperm attributes for the two competing males. The blue lines represent the fit of a generalized linear mixed model with a binomial distribution of errors, and the shaded areas the 95% confidence intervals. See table 2 for sample sizes.

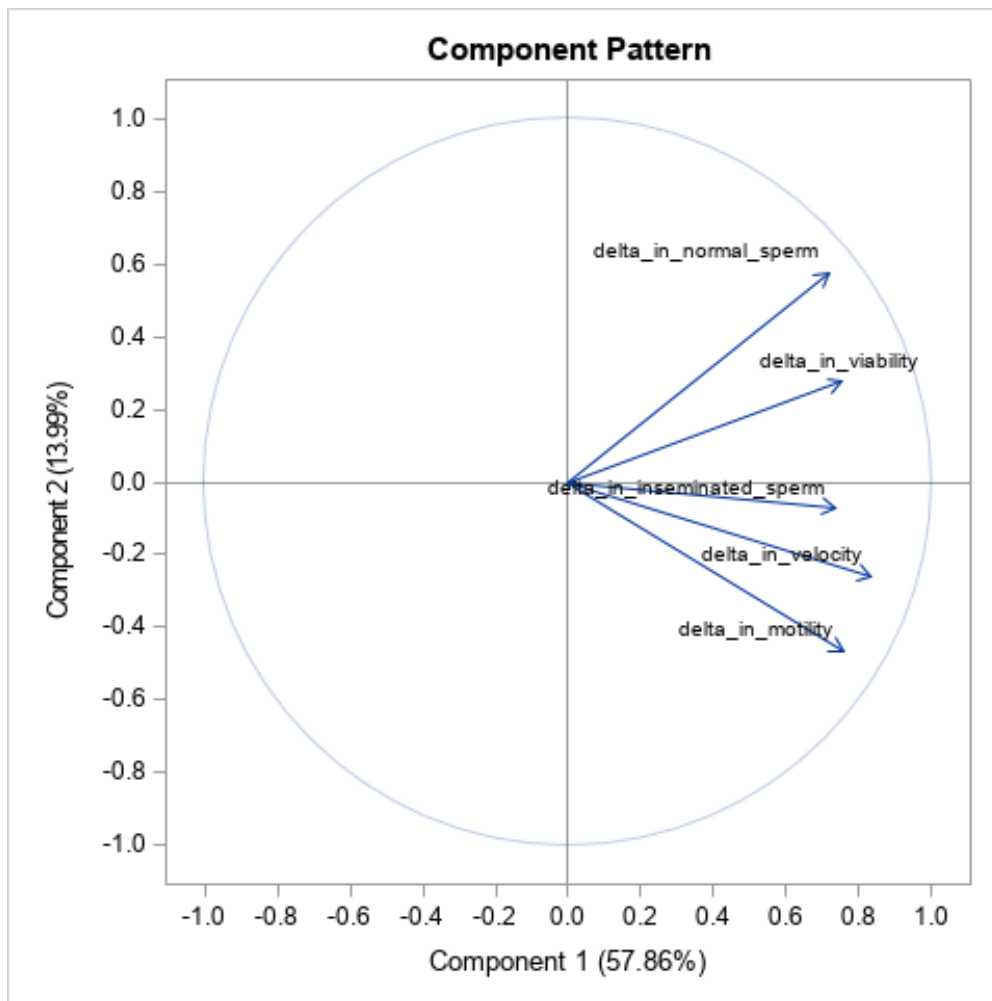


Figure 4. Pattern of loadings for the principal components 1 and 2 extracted from a PCA that included the within dyad differences in five ejaculate/sperm attributes [number of sperm inseminated, sperm motility, viability, velocity and normal morphology (N = 180 dyads)]. Component 1 accounted for most of the variance (58%) and all differences had strong positive loadings on component 1.

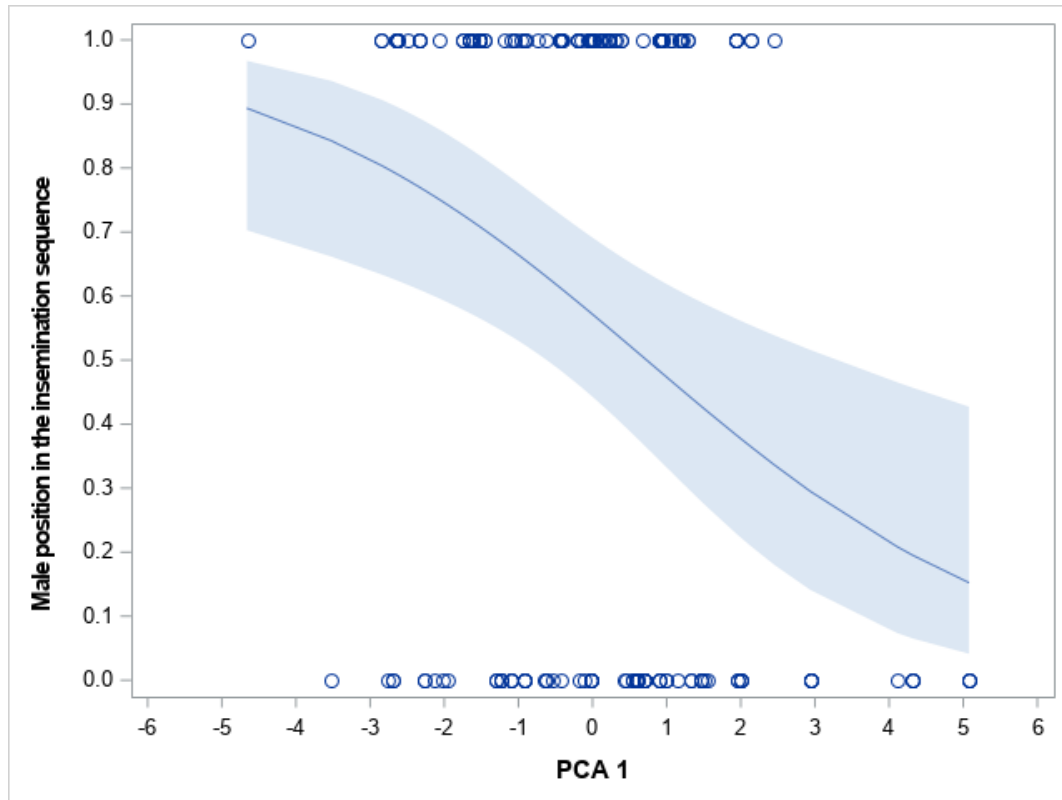


Figure 5. Relationship between the position of successful males (males that fertilized the egg) in the insemination sequence (0 = first; 1 = last) and the component 1 extracted from a PCA that included the within dyad difference in five ejaculate/sperm attributes (the percentage of sperm with normal morphology in the ejaculate, the percentage of motile sperm in the ejaculate, the number of inseminated sperm and sperm curvilinear velocity). The blue line represents the fit of a generalized linear mixed model with a binomial distribution of errors, and the shaded areas the 95% confidence intervals. N = 72, males and 180 dyads.

DISCUSSION

This thesis work aimed to investigate reproductive senescence and post copulatory selection mechanism in the North African Houbara Bustard. In order to achieve this, we had three main objectives. The first one was to gain a better understanding of houbara reproductive biology. This was done in chapter one by exploring anatomy and histology of male and female reproductive tract during and outside of the breeding season and describing sperm ultrastructure and morphology classification. The second objective was to investigate LMP mechanism, and it was achieved in chapter 2 by evaluating SSTs after inseminations with fluorescent sperm. The third objective was to explore male reproductive senescence and its effect on post copulatory selection, and this was divided in four chapters. In chapter 3 we investigated testosterone levels pattern across season in relation to male age. With chapter 4 we explored male age impact on sperm traits and possible trade-off between sperm number and quality, multiple aspects of sperm function were analysed. The objective of chapter 5 was to assess male age, sperm age effects and their possible interaction on sperm quality and reproductive success. Finally, in chapter 6 we investigated LMP in controlled experimental conditions to disentangle the importance of male age, mating order, and sperm quality in sperm competition outcome.

Anatomy and histology of male and female reproductive tracts, as well as a classification of sperm morphological abnormalities have been described and documented for the first time in Otidiformes. A regression of male and female gonads and reproductive tracts was observed outside the reproductive season. During the breeding season, males exhibit larger testis and seminiferous tubule diameters compared to the non-breeding season, indicating higher spermatogenic activity and sperm production. During the non-breeding season, females showed a regression of the ovary and oviduct, with only pre-hierarchical follicles visible, indicating low folliculogenesis activity. Similarly, spermatogenesis activity decreased but did not completely stop in males, with elongated spermatids and spermatozoa visible in the testis. This suggests that even low testosterone levels can support a low rate of spermatogenesis. Regression of reproductive organs has been observed in various seasonal birds, including the Japanese Quail (Artoni *et al.* 1999), African Penguin (Mafunda *et al.* 2021), Greater Rhea (Carvalho *et al.* 2015; Freneau *et al.* 2016), Red-winged Tinamou (Baraldi-Artoni *et al.* 2007), Jungle Crow (Islam *et al.* 2010), Emu (Du Plessis 2012), Yangzhou Goose (Akhtar *et al.* 2020), California Gull (Johnston 1956b, 1956a), and Guinea Fowl (Abdul-Rahman *et al.* 2016). Seasonal variation of reproductive hormones controls folliculogenesis and spermatogenesis activity (Vizcarra *et al.* 2015; Johnson 2015). The regression of reproductive organs in houbara can be explained by the seasonal secretion pattern of LH, FSH,

testosterone and progesterone (Saint Jalme *et al.* 1996). In chapter 2 we also reported lower testosterone values in the beginning and in the end of the season for male testosterone.

The normal morphology of houbara sperm is similar to that of other non-passerine species. However, its average size and number of mitochondria are lower than what is observed in other avian species (McFarlane 1963; Korn *et al.* 2000; Jamieson 2007). Compared to other bustards, it is in the same range than Asian houbara bustards, mean length of 47.9 μ m, but shorter than Arabian bustard, mean length of 59.74 μ m (Carreira *et al.* 2022). It is worth noting that houbara sperm lacks a perforatorium, and the round-shaped acrosome is directly attached to the anterior side of the nucleus, as the nuclear rostrum is also absent. A similar acrosome shape without a perforatorium has been observed in other species such as woodpeckers (Henley *et al.* 1978), jacanas (Saita *et al.* 1983) and cattle egrets (Roopnarine *et al.* 2020). The function of this sperm structure is not well understood, but it may be involved in the acrosome reaction (Jamieson 2007; Hirohashi & Yanagimachi 2018). However, exact mechanisms of avian acrosome reaction are not yet understood, but several signalling pathways have been found to play a role (Ahammad *et al.* 2013; Nguyen *et al.* 2016; Priyadarshana *et al.* 2018; Ushiyama *et al.* 2019).

The average percentage of abnormal sperm observed in houbara is 32%, which is higher than that documented in other bird species and in another bustard species, the Arabian Bustard (*Ardeotis arabs*) with an average of 90% of live and normal sperm reported (Carreira *et al.* 2022). The most common abnormalities we observed were midpiece (8%) and head defects (5%). Our results in chapters 4, 5 and 6 on the sperm characteristics highlighted its importance in reproductive success and sperm competition therefore having reliable sperm analysis techniques is necessary. The classification and description of the different sperm defects we provided in chapter one allowed us to precisely evaluate houbara sperm morphology in the next chapters. Assessing sperm morphology is not only important regarding fertility but it can also provide information on defective spermatogenesis. Indeed, sperm morphology abnormalities can be attributed to spermatogenesis dysfunction but can also arise after spermatogenesis. The occurrence of sperm multiple flagellum, a defect we here classified as “teratogenic sperm”, can be caused by two potential mechanisms in mammalian sperm. A disruption of meiotic division during spermatogenesis may lead to the fusion of two or more potential sperm (diploid or polyploid cells). Macrocephalic sperm with multiple tails, reported in various animals and man (Barth & Oko 1989; Weissenberg *et al.* 1998; Lewis-Jones *et al.* 2003; Perrin *et al.* 2008; Revay *et al.* 2010), would be the consequence of this defective meiosis. Another suggested origin of multiple flagellum is an abnormal formation of centrioles during spermiogenesis resulting in multiflagellate sperm (Bartoov *et al.* 1980; Escalier 1983; Lewis-Jones *et al.* 2003). Both of these disruptions seem to occur in emus as both macrocephalic multiflagellate sperm and multiflagellate sperm of normal head

dimensions can be observed (Du Plessis & Soley 2012). It is also likely to cause teratogenic sperm defects we observed in houbara sperm, as we here reported both double head and double flagellum sperm and normal head and double flagellum sperm. Head defects can have several origins. Underdeveloped head we reported, also described as round head in emu sperm (Du Plessis & Soley 2012) could be due to defective formation of the circular manchette which initiate and sustain nuclear elongation during spermiogenesis (Soley 1997). Bent heads might arise from defective chromatin compaction during spermiogenesis as suggested by poorly compact chromatin in bent head in TEM observations in emus and ostriches (Du Plessis & Soley 2011; Du Plessis *et al.* 2014) but also in humans (Zini *et al.* 2009). However, bent heads are sometimes associated with cytoplasmic droplet (Du Plessis & Soley 2011) implying an uncomplete removal of residual cytoplasm drop during spermiation. Another cause of this head defect is a distension of the plasmalemma due to osmotic shock, for example in contact with hypotonic extender and this can also be associated with various tail defects (Bakst & Sexton 1979). Chapter 1 results revealed that abnormal midpiece are the most common sperm defect in houbara, this defect could be due defective alignment of mitochondria during the maturation phase as our TEM pictures revealed disorganized mitochondria.

The investigation of the female anatomy enabled the identification of the site of sperm storage (SSTs). The mucosa folds of the UVJ were dissected under the stereomicroscope, and observation under the microscope revealed the presence of SSTs similar to those of other avian species (Birkhead *et al.* 1990; Bakst 1994, 1998; Blesbois & Brillard 2007). SSTs-like structures were also located in the infundibulum mucosa; however, they appeared less abundant than those of the UVJ, and stored sperm were never observed inside these SSTs. SSTs from the UVJ are often referred to in the literature as the primary storage site, but sperm can also be stored in SST located in the mucosal folds of the infundibulum (Blesbois & Brillard 2007; Hemmings *et al.* 2015). The abundance of SSTs in houbara was not quantified, but it was visually clear that their number depends on the individual, and possibly the female reproductive status (SST maturity and egg-laying delay). Hemmings *et al.* (2015) studied the timing of insemination and sperm storage in domestic hens. They found that sperm are stored from 8 to 10 days before ovulation, mainly in the SSTs from the utero-vaginal junction (UVJ). However, in maturing hens 1 to 6 days pre-laying, the number of sperm stored in SSTs from the infundibulum increased tenfold and was associated with a peak of plasmatic progesterone. The maximum number of sperm was stored in all SSTs 6 to 8 days before ovulation, which was associated with a peak of plasmatic oestradiol (Hemmings *et al.* 2015). The timing of sperm acceptance inside SSTs appears to be associated with oviduct and SSTs maturation, under hormonal control, but is also species dependent. In turkeys, for example, sperm can be accepted up to 2 to 3 weeks before the onset of egg laying, and they can also be stored for up to 72 days, which is longer than in other species (Lorenz

1950). In houbara, optimal fertility results are achieved by inseminating females 3 to 6 days before egg laying, with peak fertility occurring at day 4 before laying (Saint Jalme *et al.* 1994). Therefore, it can be assumed that this timing is also associated with optimal sperm storage capacity. Several studies reported that spermatozoa enter a quiescent state inside SSTs (Bakst 1987; Matsuzaki *et al.* 2015; Matsuzaki & Sasanami 2022) but other suggested a rheotaxis model, where sperm stay motile and swim against a fluid current generated by SSTs epithelial cells (Froman 2003; El-Sherry *et al.* 2022). Our observations are consistent with the quiescent model, during observation of fresh tissue where mucosa cilia were still moving, sperm stored inside SSTs were immotile whereas some loose sperm with low motility were sometimes observed close to the UVJ mucosa. The mechanisms of avian sperm motility inactivation and activation inside the oviduct are not fully understood. Lactic acid and may be involved in sperm inactivation whereas progesterone and HSP70 in re-activation (Matsuzaki & Sasanami 2022). Further investigations are needed to elucidate mechanisms responsible of motility regulation in houbara.

Identifying the location of the SSTs allowed us to investigate the mechanism of LMP. We used fluorescent staining techniques to visualize and identify sperm cells of different males inside the SSTs. This technique is challenging and requires prior *in vitro* testing to ensure that the fluorochromes do not alter sperm motility, stain all sperm cells inside the ejaculates, and provide a sufficient fluorescent signal over time in the oviduct environment. The method of labelling sperm was initially used in insects to investigate storage dynamics during sperm competition. This was achieved by creating transgenic lines that produce sperm expressing fluorescent proteins, which can be easily observed under a microscope and allows for the differentiation of sperm from different males (Price *et al.* 1999; Manier *et al.* 2010; Scolari *et al.* 2014). In the context of captive breeding for wild birds, the use of transgenic lines is not feasible. Instead, fluorescent staining of sperm cells must be performed in ejaculated sperm, before insemination. To date, only three molecules have been successfully used *in vivo* in birds: Hoechst 33342 (Bakst 1994; McDaniel *et al.* 1997; King *et al.* 2002; Matsuzaki *et al.* 2021; Matsuzaki *et al.* 2023), pHrodo-Red AM (Matsuzaki *et al.* 2021; Matsuzaki *et al.* 2023) and MitoTracker™ Red FM (Chapter 2). We tested several fluorescent stains *in vitro* before defining the final protocol for the experiment. In addition to Hoechst 33342, we also tested NUCLEAR-ID® Red DNA staining, acridine orange (Invitrogen™, US) and nuclear green (Abcam, UK) were found to either alter motility or provide a poor fluorescent signal and were therefore not considered as potential candidates for *in vivo* use. Although using two nuclear stains would be ideal, we were able to differentiate between sperm from two males by using a combination of a nuclear stain, Hoechst 33342, and a mitochondrial stain, MitoTracker™ Red FM.

By identifying the sperm of different males after competitive inseminations, we showed that active sperm displacement is likely the main mechanism underlying the last male advantage in houbara. While passive sperm loss is often described as the main mechanism responsible for LMP in birds, only a few studies have used techniques to visualize and differentiate sperm of different males inside SSTs. Sperm segregation has been observed in fowls and turkeys (King *et al.* 2002), while a more recent study supports sperm displacement in fowls (Matsuzaki *et al.* 2023). Active sperm displacement has also been reported in fruit flies (Manier *et al.* 2010). Due to the limited research on birds, it is challenging to establish a general pattern of LMP mechanism, which may vary depending on the species. It is important to note that although no sperm loss was observed 72 h after insemination, passive sperm loss does occur in Houbara, as fertility declines with time after a single insemination, as reported by Saint Jalme *et al.* (1994).

Testosterone levels were reported in male breeders from December to June. The levels progressively increased from the first collection to reach a maximum level at the end of February and then declined. This peak of testosterone secretion preceded the peaks in sperm production and displaying frequency, which were reached 30 days later, at the end of March. Testosterone fluctuations across the breeding season were expected, as similar hormonal patterns have already been recorded in houbara males (Saint Jalme *et al.* 1996). Our study found testosterone concentration values within the same range as those previously recorded, although the peak of testosterone was observed later in our study compared to the ones of birds housed in Saudi Arabia (Saint Jalme *et al.* 1996). Thus, previously the reported maximum LH and testosterone concentrations were found in January, at the beginning of the displaying period, when body mass was also at its highest. Testosterone levels were correlated with the percentage of males displaying, the different locations of the studies, Saudi Arabia (Saint Jalme *et al.* 1996) and Morocco (Chapter 3), may explain the difference in the timing of peak testosterone.

The onset of reproduction can be influenced by temperature, weather conditions, and photoperiod (Wingfield *et al.* 1983; Paster 1991; Dawson *et al.* 2001). A decrease in body mass was also observed, most likely due to the energetic cost of displaying. Houbara males can display for several hours during the day (Hingrat *et al.* 2008). The peak of testosterone at the beginning of the breeding season may initiate sexual behaviour and sperm production, as the frequency of displays and sperm count increased and reached maximum values 30 days later. Although testosterone concentrations declined after February, levels remained sufficient to maintain sperm production and display activity throughout the season. Our histological study of testicular epithelium during the non-breeding season (September) in chapter 1 also suggests that a low testosterone concentration can maintain slow

spermatogenic activity. Late germ cells, elongated spermatids and spermatozoa were observed in the seminiferous tubules, although they were less numerous compared to the breeding season.

Other bustard species have also been reported to exhibit similar seasonal patterns of testosterone. In the Great Bustard, the faecal concentration of testosterone was highest at the beginning of the display period, and testosterone levels were found to be correlated with display activity (Biczó & Péczely 2007). Testosterone peaks were observed during the day, specifically in the early morning and late afternoon when males were displaying. In Kori bustards, testosterone levels increased with the onset of reproductive behaviour and remained elevated throughout the breeding season (Penfold *et al.* 2013). This increase in testosterone may be associated with the establishment of displaying territories and high levels of male interactions and aggressive behaviours in the wild. However, such behaviours were not recorded in our studies as male breeders were kept in individual cages. Though, male breeders could observe the displays and hear the vocal signals of other males. In great bustards, interactions between males and aggressive behaviour have been reported at the beginning of the breeding season (Biczó & Péczely 2007). In black grouse (*Tetrao tetrix*), a species with a lek mating system, testosterone is likely to influence the establishment of territories, as males with higher levels of androgens were located in the central area of the lek and were preferred by females (Harding 1999).

Since sperm quality and reproductive success are negatively affected in aging males, an age-related decline in male testosterone production might be expected in houbara. This phenomenon has been observed in several bird species, including the Florida Scrub-jay (*Aphelocoma coerulescens*) (Wilcoxon *et al.* 2013), the Japanese Quail (Balthazart *et al.* 1984), the Common Tern (*Sterna hirundo*) (Nisbet *et al.* 1999), the Pied Flycatcher (*Ficedula hypoleuca*) (Moreno *et al.* 2014), the Barn Swallow (*Hirundo rustica*) (Adámková *et al.* 2019), and the Great Tit (Těšický *et al.* 2022). In great tits, testosterone levels are associated with secondary sexual characteristics. Males with high concentrations of testosterone have significantly brighter carotenoid-pigmented yellow plumage (Těšický *et al.* 2022). A decrease in testosterone levels may reduce the chances of mating by affecting the expression of secondary sexual characteristics.

Contrary to our prediction, the levels of testosterone were found to be similar in both young and old male breeders. One possible explanation for this is the involvement of testosterone in sexual display activity. The lack of impact of male age on display activity (Preston *et al.* 2011), and its positive correlation with testosterone levels (Saint Jalme *et al.* 1996), likely explains the absence of an age-related decline in testosterone concentration in houbara. Similarly, in the tropical lekking bird, the Wire-tailed Manakin (*Pipra filicauda*), testosterone levels did not decline with age and were associated

with male social status. Dominant males had higher levels of testosterone, and the authors suggested that testosterone was involved in establishing male dominance hierarchies (Ryder *et al.* 2011).

It should be noted that this experiment was conducted on a small sample size of birds that were born in captivity and were part of the breeder flock. Semen collections were performed every two days during the breeding season, and the birds were kept in individual cages. However, they were able to see other males displaying and hear booming calls. No data on mating frequency has been reported in nature. However, during the breeding season, males display in specific sites that are separated by an average distance of 550 meters from other males. Displays are observed early in the morning and late in the afternoon and can last for several hours (Hingrat *et al.* 2008). Therefore, wild males may have different testosterone levels compared to captive males.

Previous research has shown male age negative impact on sperm quality (Preston *et al.*, 2011), reproductive success and survival in houbara (Preston *et al.* 2015; Vuarin *et al.* 2019a). The results of chapter 5 were consistent with previous findings and showed a negative effect of male age on sperm quality and reproductive success. A decrease in sperm count, ejaculate volume, sperm motility, and velocity were observed. This decrease in sperm quality was reflected in the lower growth and survival rates of chicks sired by older males. This negative impact on the offspring may be attributed to sperm DNA mutations. As males age, DNA mutations are likely to accumulate in the germline due to continuous division, and such mutations can impact the offspring health (Aitken 2020). Epigenetics changes, such as DNA methylations, might also be involved in the offspring growth and survival decrease. In mammals, age related changes in DNA methylation levels have been observed and are involved in gene networks controlling embryo developmental, neurodevelopmental, growth and metabolic pathways (Ashapkin *et al.* 2023). In mice for example, paternal age is associated with decreased embryonic placental weight, foetal weight and length and with hypermethylation of placental DNA imprinting region (Denomme *et al.* 2020).

We expected that, due to prolonged storage in the male epididymis/vas deferens, sperm would be subject to senescence when collected at longer time intervals. However, contrary to this prediction, we found that sperm number and quality increased with longer collection intervals. Specifically, males collected on day 5 and 10 exhibited higher sperm number, volume, motility, velocity, viability, and morphology. ROS production increased, but it did not appear to affect sperm function. The effects on sperm quantity and quality were mirrored by higher hatching success. It seems that during their transit inside the ducts, spermatozoa undergo some kind of maturation process rather than senescence. Sperm senescence after extended storage inside the male's ducts has already been reported. Studies have shown that prolonged sperm storage can have negative effects on reproductive success in various

species. For example, in guppies, decreased velocity and impaired fertilization have been observed (Gasparini *et al.* 2014; Gasparini *et al.* 2017). Similarly, in zebrafishes, reduced viability and increased DNA fragmentation have been reported (Cattelan & Gasparini 2021). In kittiwakes, decreased fertilization, embryo development, hatching, and chick condition have been observed (White *et al.* 2008). In our study we only analysed the sperm component of the ejaculate and not the seminal plasma. As the ejaculate volume increased with male storage time, it is also likely that the seminal plasma composition also changes and influence the sperm quality. Evidence of differential seminal plasma composition associated with fertility have been reported before. Turkey males with yellow semen syndrome have reduced sperm viability, fertility and hatching success and present different seminal plasma proteome and metabolite compositions compared to healthy males (Slowinska *et al.* 2018; Słowińska *et al.* 2019). In chickens, low and high motility sperm present different seminal plasma proteomic profiles (Li *et al.* 2020). Moreover, in chickens, males with higher sperm motility and fertility had seminal plasma with higher proportion of extracellular vesicles and these vesicles contained different protein markers (Cordeiro *et al.* 2021). Thus, the sperm quality increase we observed as well as the higher hatching rate associated might be associated with changes in seminal plasma composition.

Histological observations of the epididymal ducts revealed the presence of round cells, possibly round spermatids, or cytoplasmic droplets. This indicates morphological maturation during transit through the male duct. Contrary to the findings of Cattelan and Gasparini (2021), a previous work, also performed in zebrafishes, found that aged sperm had a higher velocity compared to freshly produced sperm (Vega-Trejo *et al.* 2019). The role of sperm maturation in the epididymal and deferent ducts in birds is still poorly understood. Sperm transit through the epididymis is rapid in birds due to their shorter epididymis compared to mammals and high rate of sperm production. It is worth noting that AI with testicular sperm does produce fertile eggs in poultry, suggesting that epididymal maturation is not mandatory for egg fertilization (Munro 1938; Howarth 1983). However, it has been suggested by several studies that maturational changes also occur in birds. For example, research has shown that sperm motility and/or velocity increase during transit from epididymis to vas deferens in domestic fowls (Munro 1938; Ahammad *et al.* 2011a), Japanese quails (Clulow & Jones 1982) and rheas (Bezerra *et al.* 2023). Additionally, in poultry, sperm viability and fertilization capacity gradually increase from the testes to the vas deferens (Ahammad *et al.* 2011b, 2011a). Bernal *et al.* (2022) reported evidence of increased sperm chromatin compaction during vas deferens transit in roosters.

The higher levels of ROS at longer storage times, without any apparent effect on sperm quality, suggest some kind of protective system against OS. This may be related to the presence of melanin in the testes. Several studies in non-avian species have suggested a possible function of melanin pigment

against OS. In bats, melanin is present around the testes and epididymis, suggesting that it protects mature sperm from UV radiation and oxidative stress during storage in the male ducts (Delmore *et al.* 2018). In frogs, melanin protects thermosensitive organs. Exposure to UVR causes an increase in internal melanin pigmentation (Franco-Belussi *et al.* 2016; Franco-Belussi *et al.* 2017). In the Mexican lizard, the testis pigmentation (inside the tunica albuginea) is unilateral, with only the active testis being coloured. The correlation between melanin pigmentation, spermatogenic activity, and ST diameter also suggests a possible role in the thermal physiology of this species (Guillette *et al.* 1983). In birds, a positive correlation has been found between testes melanisation and mtDNA mutations, suggesting an adaptive function related to protection against oxidative stress (Galván *et al.* 2011). However, male Indian robins only have pigmented testes during the non-breeding season and show reduced spermatogenic activity. The authors have suggested that testes melanisation may be due to adrenal dysfunction affecting spermatogenesis. Future research would be of interest to gain more insight into the function of testicular melanisation.

We were expecting a similar pattern of reproductive senescence in the experiments of chapters 4 and 6 than the one observed in chapter 5. However, the results of these chapters were not always consistent with our predictions and did not show the exact same pattern. While older males had lower sperm count and ejaculate volume in chapter 4, all other sperm parameters showed no difference between age classes, including motility and velocity. Due to the difference in sperm count, we anticipated a higher rate of fertilization in young males during the experiment in chapter 6. This was also supported by a previous study which showed that siring success was skewed towards young males when females were inseminated with a mix of young and old males' sperm (Vuarin *et al.* 2019a). However, when females were inseminated in sequence with ejaculates from young and old males, fertilization success was similar for both young and old males. It is surprising that we did not observe the same pattern of reproductive senescence between Chapter 5 and Chapters 4/6 and previous experiments.

Different experimental conditions were used in these experiments. Chapter 5 involved inseminating females with a single diluted ejaculate from males of two age classes: young males aged 3-6 years old or old males aged 12-16 years old. In this study sperm samples were selected based on a threshold of motility score and concentration and males were respectively collected in the 3 intervals: 1, 5 or 10 days after an initial collection to achieve different storage time inside the male ducts and thus different range of sperm aging. Additionally, the same ejaculates were used for the experiments in chapters 4 and 6 with different objective, but different for chapter 5. Chapters 4 aimed to investigate trade-off between sperm quantity and quality and chapter 6 aimed to disentangle the effect of male age, sperm quality and insemination order on paternity success. For this, we aimed to replicate the

natural mating conditions and therefore we used different experimental conditions than the ones of chapter 5. Firstly, we increased the age gap between young males (2 to 5 years old) and old males (14 to 20 years old) to enhance age-related differences. Secondly, we inseminated females in sequence with raw ejaculates of two males, without controlling for motility and sperm count. However, we had to set a minimum volume threshold of 80 μ l due to the captive program's AI requirements. Indeed, AI cannot be performed below 50 μ l and 30 μ l were necessary for sperm trait analysis. By applying this threshold, we selected males with larger ejaculate volumes from the breeding flock. As we have shown that sperm number is correlated with volume and several quality traits, we may have indirectly selected males with higher sperm quality. Therefore, the criterion for ejaculate volume used likely explains why we did not observe a more obvious senescence pattern in this experiment.

In polyandrous species, when females mate with multiple males, sperm competition occurs. The number of inseminated sperm, as well as their quality, including viability, motility, and morphology, can predict fertilization success. In this context, under high risk of sperm competition, two evolutionary responses could be observed. Sperm quantity and quality traits could be expected to co-evolve to maximize fertility as their complementary roles predict fertilization success and competition outcome. However, as investment in sperm production is energetically costly, a trade-off between sperm numbers and sperm quality traits is expected. Interestingly, contrary to our initial prediction, the number of sperm in the ejaculate was positively correlated with several sperm quality traits. Our results of Chapter 4 suggest that males may invest in both sperm quantity and sperm quality. Several studies among birds, mammal and invertebrates have reported positive correlation between sperm competition, sperm number and sperm quality. In a wild red deer population, sperm count was positively correlated with several different sperm quality traits, sperm motility, velocity, normal morphology, viability and acrosome integrity (Malo *et al.* 2005). In 8 monogamous avian species with different levels of extra pair copulation of Australian Maluridae family, sperm competition levels were associated with increases of sperm quantity, proportion of testicular spermatogenic tissue and sperm quality traits (Rowe & Pruett-Jones 2011). Another study on 18 rodent species found positive covariation between sperm number and proportions of normal sperm, acrosome integrity and motile sperm (Gómez Montoto *et al.* 2011). Degueldre and Aron (2023) reported in nine swallow desert ants positive correlation between sperm competition, sperm production and sperm viability. (Liao *et al.* 2019) found a strong positive correlation between sperm number in the ejaculate and sperm viability and normal morphology in a large dataset of sperm traits from 20 bird species of the Phasianidae family. In the same study, it was found that sperm number positively correlated with sperm storage duration in the female tract. Additionally, polyandrous species had significantly higher sperm number, ejaculate volume, and sperm viability compared to monogamous birds (Liao *et al.* 2019).

There may be several explanations for the absence of a trade-off between sperm quantity and quality. The first one is both sperm quantity and quality have been selected under sperm competition and sperm traits to coevolved to maximise fertility as suggested by the studies mentioned above. Another possibility is, as suggested by van Noordwijk and Jong (1986), positive correlations between life history traits can be expected when individuals differ more in their capacity to acquire resources than in their allocation rule to conflicting functions. This phenomenon can account for positive correlations in free-ranging animals that differ in their access to resources when living in variable and unpredictable environments. However, male houbara bustards kept in captivity are subject to uniform environmental conditions and fed ad libitum. Therefore, it is unlikely that the positive correlations reported here could be due to significant individual variation in the amount of available resources. Another possible explanation for the observed result is that, since post-copulatory selection occurs in the breeding facility (females can be inseminated with sperm from different males), males with high levels of different sperm quality traits may have a significant competitive advantage (Sorci *et al.* 2023). Thus, although efforts were made to ensure the representativeness of breeders, traits related to sperm quality and volume may act as selective barriers in captivity. These traits are not counterbalanced by environmental selection, as would be expected in the wild. The favourable environmental conditions, with abundant resources, may have alleviated the constraints or energetic costs that prevent positive correlations from expressing in harsher natural environments, ultimately resulting in a positive correlation between sperm quantity and quality.

Chapter 6 provided strong evidence of last male precedence (LMP), with approximately 60% of offspring being sired by the male in the last position of the insemination sequence. Our model shows that the strength of LMP remains unchanged when sperm traits are taken into account. Males with poor quality ejaculates were able to fertilise eggs when they were in the last position, provided there is a certain threshold for sperm parameters (20% difference in motile and normal morphology sperm). Our findings align with a previous report that demonstrated insemination order and the delay between insemination and laying as the strongest predictors of siring success in houbara (Sorci *et al.* 2023). Additionally, our results support the hypothesis that the combined effect of last male precedence and random mating sequence may contribute to maintaining variation in traits that are believed to be under strong selection. This implies that sperm traits are heritable, as has been observed in various species, including the North African Houbara Bustard (Chargé *et al.* 2013). However, contrary to our predictions, male age did not affect the strength of LMP, and siring success did not differ between age classes. This result was unexpected, as houbara sperm quality typically declines with age (Preston *et al.* 2011) and paternity is biased towards young males when females are inseminated with a mix of sperm from young and old males (Vuarin *et al.* 2019a). Upon comparing the sperm parameters of

competing males within the dyads, it was observed that old males had a lower number of inseminated sperm, but all other parameters were similar. The absence of an age impact on LMP effect may be explained by the ejaculate volume threshold used for AI, since only males producing ejaculates above 80 microlites were used. Another limit of this experiment is that paternities could not be attribute for early embryo death as only chicks or dead embryo with enough material could be sampled. In these conditions we do not have data on early embryonic death and the potential effect of age on embryonic development.

Despite the occurrence of LMP, 40% of the offspring were sired by the first male. In such cases, a significant difference in sperm quality (motility and morphology) was observed within the dyad, upon reaching a certain threshold. Therefore, sperm quality can also predict the outcome of sperm competition. Similarly, Sorci *et al.* (2023) reported that the number of inseminated sperm and their motility were predictors of siring success, although less important than the insemination order. In terms of the mechanism, poor quality sperm may not be able to displace sperm that is already stored in the SSTs, resulting in only a small number of sperm being stored. Alternatively, this low-quality sperm may be able to displace sperm from previous males but would have a lower chance of reaching the fertilization site. The outcome of both scenarios would be a reduced success in fertilization. Matsuzaki *et al.* (2021) showed that SST filling capacity was associated with fertilization success in competitive inseminations. They inseminated females with fluorescent stained sperm mix of two males with different sperm phenotypes and reported that SSTs filling, and fertilization was biased toward sperm with longer midpiece and flagellum.

It is important to recall that pre-copulatory selection could not operate due to experimental and captive breeding constraints, as females were artificially inseminated. However, in some species, females can influence sperm competition depending on precopulatory signals. For example, fowl females can eject sperm depending on male's dominance status (Pizzari & Birkhead 2000; Dean *et al.* 2011). In crickets (*Teleogryllus oceanicus*), females can avoid inbreeding by storing less sperm from related males (Tuni *et al.* 2013). Additionally, in the guppy, a polyandrous fish, female perception of male attractiveness can influence post-copulatory selection, as females retain more sperm from colourful males (Pilastro *et al.* 2004). Gasparini & Evans (2018) demonstrated stronger paternity biases in natural mating compared to AI in the same species. They also found a greater opportunity for post-copulatory selection when females controlled matings. This relationship between pre- and post-copulatory selection has not been studied in houbara. However, Loyau and Lacroix (2010) investigated the influence of precopulatory visual signals on female allocation. They found that when females were stimulated by displaying males, their eggs had increased hatching success. Additionally, they allocated more androgens in their eggs, leading to increased circulating testosterone in chicks and a better

growth rate. By having control over pre-copulatory selection, female houbara bustards may influence the outcome of post-copulatory selection. Allowing females to choose their mates may result in different outcomes, potentially revealing an age effect on LMP.



CONCLUSION AND PERSPECTIVES

Overall, this thesis provides new insights into various aspects of bustard reproductive biology, mechanisms of sperm storage, male reproductive aging, and post-copulatory selection. We provided new knowledge on the reproductive biology of bustards, including a first description of the macro and microscopic anatomy of male and female reproductive organs. The morphology of houbara sperm was also described, and a classification of abnormalities was established. Fifteen bustard species are red-listed, and conservation programs have been initiated for two critically endangered species: the great Indian bustard and the lesser florican (Collar 2021; Srinivas *et al.* 2022). In this context our report on reproductive biology may be of value to other species of bustards. Investigating spermatogenesis would not only give information on the duration of sperm production but would also provide further information regarding sperm morphology. Indeed, ultrastructure observations of the seminiferous epithelium of the testis can give insights on the origin of morphological defects that can arise from different steps of spermatogenesis: mitotic/meiotic divisions, spermiogenesis, spermiation. Morphology evaluation of sperm collected at different location of male ducts could also provide information on a possible morphology maturation through epididymal transit. Investigating potential age-related impact on reproductive tract anatomy and histology would provide additional information on houbara reproductive aging. Moreover, exploring the function of melanin pigment in reproductive organs and its possible relation with oxidative stress and sperm quality would be of interest.

The exploration of the mechanisms of last male precedence revealed that active sperm displacement is likely the primary mechanism in houbara. These findings provide new insights into the mechanism of sperm competition in birds, as studies using direct labelling methods are rare. Additionally, we successfully used two fluorochromes to visualize the sperm of different males inside the storage site. This technique could be useful for investigating poorly understood aspects of sperm storage and competition in birds. Specifically, it could be used to explore the entrance, maintenance with possible binding sites, and release of sperm inside the SSTs, as well as the potential hormonal and molecular regulation. In addition, it would be interesting to investigate the control of sperm motility during storage, as it is known that sperm enter a quiescent state once stored in SSTs, but the processes of inactivation and reactivation are not fully understood. For this, studying sperm metabolism would be useful, the relative importance of glycolysis and oxidative phosphorylation in relation to motility could be evaluated. The timing between insemination, optimal storage, and oviposition could also be studied. Although there has been little research on sperm competition within the egg, the use of a



fluorescent staining method would allow for further investigation of this phenomenon following sequential inseminations.

Regarding reproductive senescence, we found varying results. While testosterone levels fluctuated throughout the breeding season and are likely to initiate sexual behaviour and sperm production, they were not affected by male age. However, we did observe a decline in sperm number, volume, motility and velocity that was dependent on male age. This impairment resulted in reduced chick growth and survival. These findings are consistent with previous studies (Preston *et al.* 2011; Preston *et al.* 2015). Further research could investigate the potential epigenetic mechanisms that impact growth rate and survival chances by identification of epigenetic signatures of old fathers and of their offspring.

We found no evidence of sperm senescence with increasing sperm age. On the contrary, longer sperm storage time inside male ducts improved both the quantity and quality of sperm, suggesting a potential maturation process. When males were collected with longer time intervals, there was an increase in sperm number, volume, motility, velocity, viability, and normal morphology, which resulted in a higher hatching success. Sperm collected on day 5 exhibited superior quality compared to those collected on day 10. Therefore, it would be valuable to investigate other collection timings to determine the optimal interval for obtaining the best sperm quality. This research could have implications for captive breeding management. Seminal plasma can also influence sperm physiology and fertility; however, nothing is known about houbara seminal plasma components. Therefore, analysis of its composition would bring further information on sperm biology. Comparing its composition between different collections, age classes and sperm quality could allow the identification of molecules involved in sperm metabolism and in maturation and aging processes.

Contrary to our initial expectations, we found positive covariations between sperm number and various sperm quality traits. This suggests that males invest in both sperm quantity and quality. Of all the traits measured, only sperm number and ejaculate volume were lower in older males. Additionally, age did not affect the correlation's strength. The observed positive covariation may be attributed to the selective breeding conditions in which high-quality sperm traits can be selected in the absence of environmental selection. It is important to note that the reported correlations are phenotypic and not genetic. (Chargé *et al.* 2013) found a genetic correlation between male display and sperm quantity, but not with sperm quality. However, their study only explored two traits related to sperm quality, namely sperm viability and morphology, which were measured using different techniques than those we used. Therefore, it is possible to expect genetic correlations between sperm number and quality by using different sperm quality parameters.



We confirmed that insemination order is a strong predictor of fertilisation success, as 60% of the chicks were sired by the male in the last position in the insemination sequence. Male age did not influence the strength of this last male precedence effect. However, these results need to be interpreted with care as paternity results were only based on a few microsatellite loci. Additionally, the volume threshold we applied may have reduced the differences between age classes.

Overall, this thesis provided a better understanding of houbara reproductive biology and new insights on reproductive aging and post-copulatory selection mechanism.

RESUME EN FRANÇAIS

Biologie de la reproduction et impact de la senescence sur la sélection sexuelle post-copulatoire chez un oiseau polyandre sauvage, l'outarde Houbara d'Afrique du Nord (*chlamydotis undulata undulata*)

Introduction

La polyandrie est un système d'accouplement courant dans la nature et désigne la tendance des femelles à s'accoupler avec plusieurs mâles au cours du même épisode reproductif. Une conséquence directe de la polyandrie est la présence simultanée de spermatozoïdes de différents mâles dans l'appareil reproducteur de la femelle. La polyandrie augmente donc les possibilités de sélection sexuelle, puisqu'en plus de la compétition entre les mâles pour l'accès à la femelle (sélection sexuelle pré-copulatoire), les spermatozoïdes de différents mâles sont également en compétition pour l'accès à l'ovocyte (sélection sexuelle post-copulatoire). Différents facteurs peuvent influencer l'issue de cette compétition spermatique. A qualité égale, le nombre de spermatozoïdes inséminés est un prédicteur du succès de fécondation. Cependant, la qualité de l'éjaculat et les caractéristiques des spermatozoïdes sont très variables entre individus et la quantité des spermatozoïdes n'est donc pas l'unique facteur déterminant l'issue de la compétition. En effet d'autres paramètres spermatiques peuvent influencer les succès de fécondation, tels que la motilité, la viabilité, la morphologie ou encore l'intégrité de l'ADN. L'âge des mâles peut également influencer l'issue de la compétition ; chez de nombreuses espèces, l'âge entraîne une baisse de la fertilité qui se traduit généralement chez les mâles par un déclin de la quantité et qualité des spermatozoïdes entraînant une diminution du succès reproducteur. Lors de leur stockage dans le tractus mâle et femelle les spermatozoïdes peuvent être sujets à une sénescence

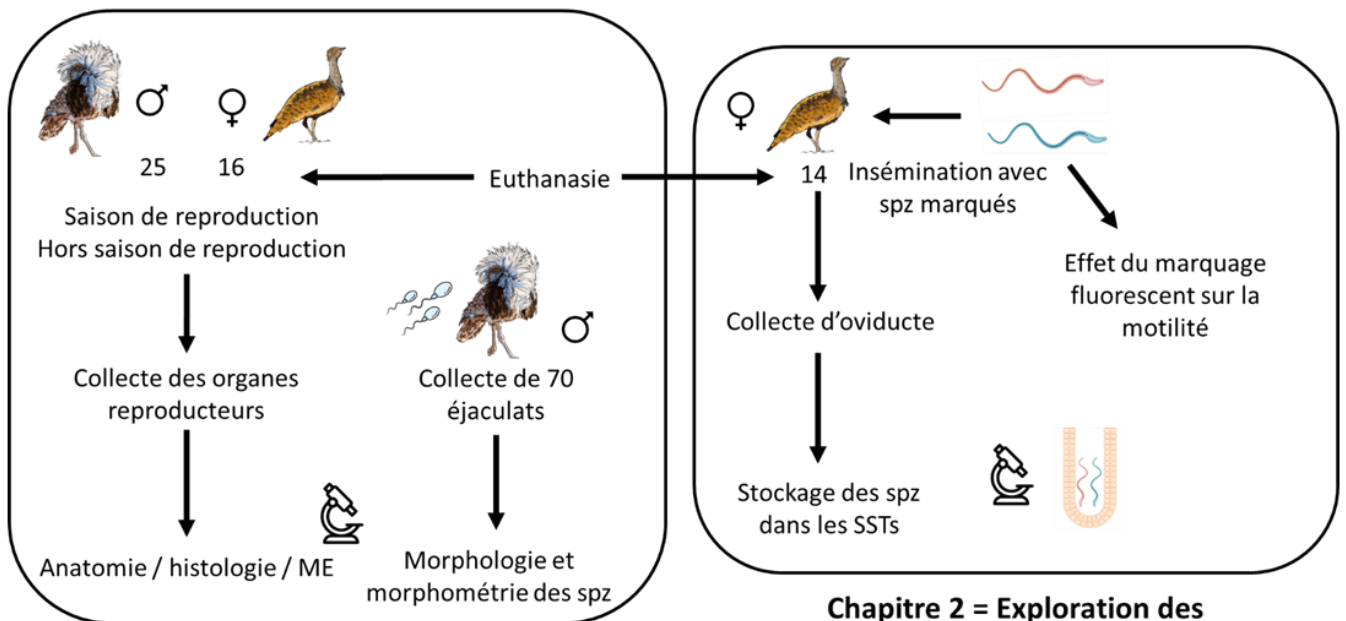
cellulaire, ce qui peut impacter leur qualité et leur capacité à féconder un ovocyte. Enfin, l'ordre d'accouplement ou d'insémination entre également en compte. Chez de nombreuses espèces d'invertébrés et d'oiseaux, cela se traduit généralement par le fait que la plupart des descendants sont engendrés par le dernier mâle, un phénomène appelé en anglais « last male precedence (LMP) » qui peut se traduire par la « préséance du dernier mâle », l'abréviation LMP sera utilisée par la suite. Chez les oiseaux, les femelles stockent les spermatozoïdes dans leur oviducte, dans des structures spécialisées appelées tubules de stockage des spermatozoïdes (SSTs); en anglais « sperm storage tubules »). Différents mécanismes de stockage ont été proposés afin d'expliquer comment le dernier mâle obtient un avantage de fécondation. Une perte passive des spermatozoïdes au cours du temps, un déplacement actif des spermatozoïdes, une stratification au sein des tubules ou encore une ségrégation dans différents tubules sont les principaux mécanismes évoqués dans la littérature. Cependant, en raison des difficultés techniques d'observation directe de tels processus, il n'existe pas de consensus sur un mécanisme prédominant et ces mécanismes dépendent probablement de l'espèce. En effet, pour étudier de tels mécanismes, il est nécessaire de disposer de connaissances sur la reproduction et l'anatomie de l'espèce étudiée.

Les outardes houbara d'Afrique du Nord (*Chlamydotis undulata undulata*) présentent un système d'accouplement de type lek explosé, où les mâles établissent des sites de parade espacés par des distances importantes. Les femelles sont polyandres et choisissent les mâles avec lesquels elles s'accoupleront. Dans ce système, il est raisonnable de supposer que la paternité confère à la progéniture des chances différentes de survie et de reproduction, de sorte que le choix du partenaire et de l'ordre d'accouplement de la femelle a des conséquences importantes sur l'évolution. Des travaux antérieurs sur l'outarde houbara ont montré que les mâles âgés souffrent de sénescence reproductive, avec une qualité de sperme et un succès reproductif réduits, et qu'ils engendrent une progéniture dont la croissance et la survie est réduite. La population captive d'outardes houbara constitue un modèle idéal pour étudier les mécanismes impliqués dans le succès reproducteur des mâles et les conséquences écologiques/évolutives de la sélection sexuelle post-copulatoire. Cette thèse s'appuie sur des travaux antérieurs menés sur l'outarde houbara et vise à approfondir notre compréhension de la biologie reproductive de l'outarde houbara, de l'effet du vieillissement sur l'issue de la compétition spermatique et du succès reproductif des mâles.

Dans une première partie, afin d'améliorer les connaissances sur la physiologie de la reproduction de l'espèce et de construire une base solide pour l'anatomie reproductive de l'outarde houbara, la morphologie des spermatozoïdes et par la suite les mécanismes de stockage dans le tractus femelle, nous avons exploré l'anatomie et l'histologie des systèmes reproductifs femelle et mâle pendant et en dehors de la saison de reproduction. La morphologie des spermatozoïdes a été décrite et une classification des différentes anomalies a été établie. Dans une deuxième partie, nous avons étudié les mécanismes sous-jacents de la LMP et la dynamique de stockage dans l'oviducte après inséminations compétitives. Dans une troisième partie, nous avons étudié la sénescence reproductive des mâles et sa relation avec la sélection post-copulatoire. Ce travail a été divisé en quatre sections afin d'explorer les différents aspects de la sénescence reproductive. Tout d'abord, les niveaux de testostérone ont été mesurés au cours de la saison de reproduction chez des mâles adultes et âgés afin de tester un éventuel effet de l'âge. Deuxièmement, pour étudier l'impact de l'âge des mâles sur les paramètres spermatiques et un éventuel compromis entre le nombre et la qualité des spermatozoïdes, plusieurs caractéristiques des spermatozoïdes ont été analysées. Troisièmement, pour tester l'effet de l'âge du mâle, de l'âge des spermatozoïdes et leur éventuelle interaction sur la qualité des spermatozoïdes et le succès de la reproduction, la sénescence des spermatozoïdes dans le tractus mâle a été explorée en contrôlant l'intervalle collecte chez des mâles jeunes et âgés. Les éjaculats ont été évalués pour divers paramètres de qualité spermatique et utilisés pour inséminer des femelles. Le taux d'éclosion, la croissance et la survie des poussins ont été suivis. Enfin, la LMP a été étudiée dans des conditions expérimentales contrôlées afin de démêler l'importance de l'âge du mâle, de l'ordre d'accouplement et de la qualité spermatique dans le succès de la fécondation. Les éjaculats de mâles jeunes et âgés ont été évalués en fonction de divers paramètres spermatiques et utilisés pour inséminer des femelles en séquence, d'abord avec mâle jeune puis âgé, et inversement, avec un intervalle de 48 heures entre les deux inséminations. Des analyses de paternité ont ensuite été réalisées sur la progéniture.

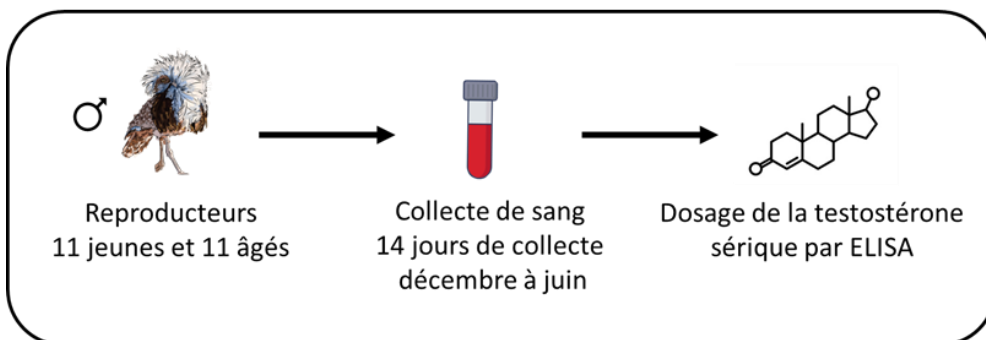
Matériel et méthodes

Ce travail de thèse s'articule en 6 chapitres dans lesquels différentes conceptions expérimentales ont été mises en œuvre pour répondre à différentes problématiques. Le schéma en Fig.1 synthétise la conception expérimentale de chaque chapitre. Les méthodes communes à ces chapitres sont présentées ci-dessous.



Chapitre 1 : Description de la morphologie des spermatozoïdes, de l'anatomie et de l'histologie des tractus reproducteurs mâle et femelle

Chapitre 2 = Exploration des mécanismes sous-jacent de la LMP



Chapitre 3 = Concentration de testostérone au cours de la saison de reproduction en fonction de l'âge

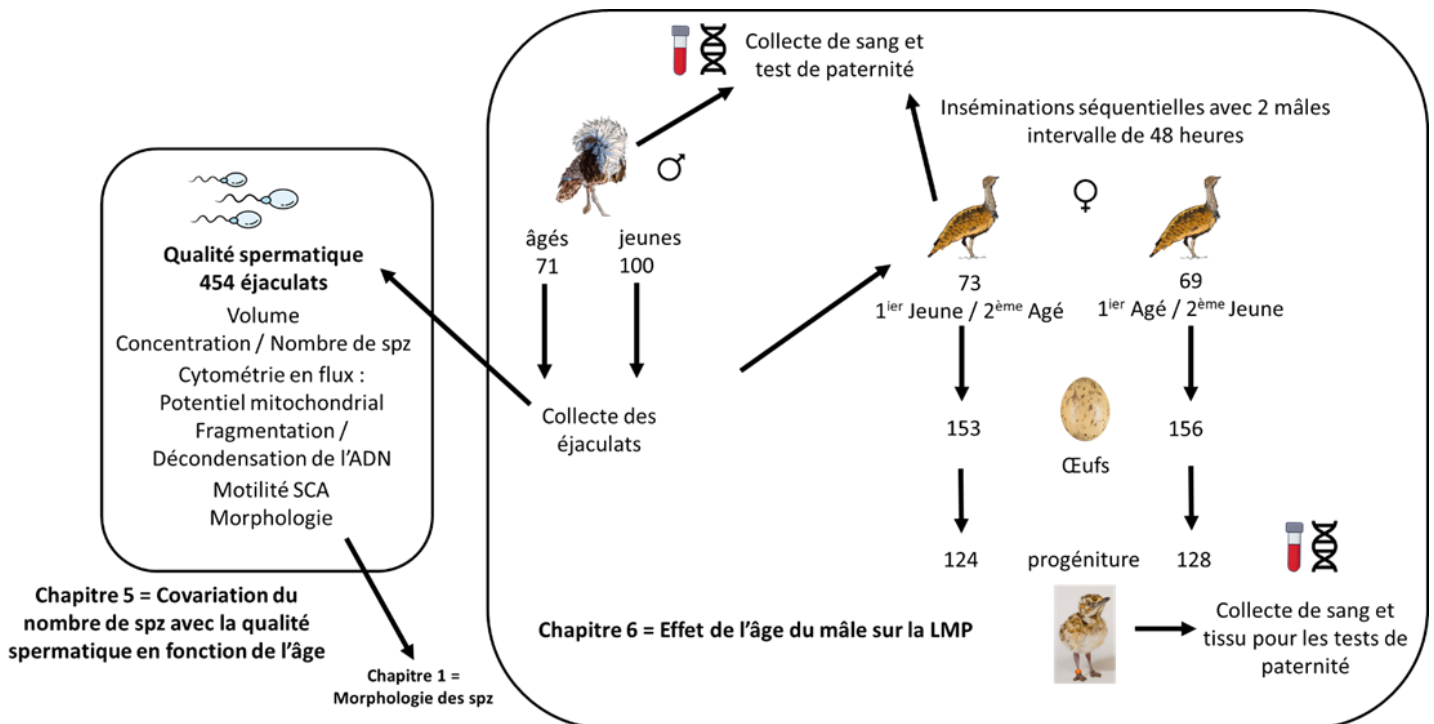
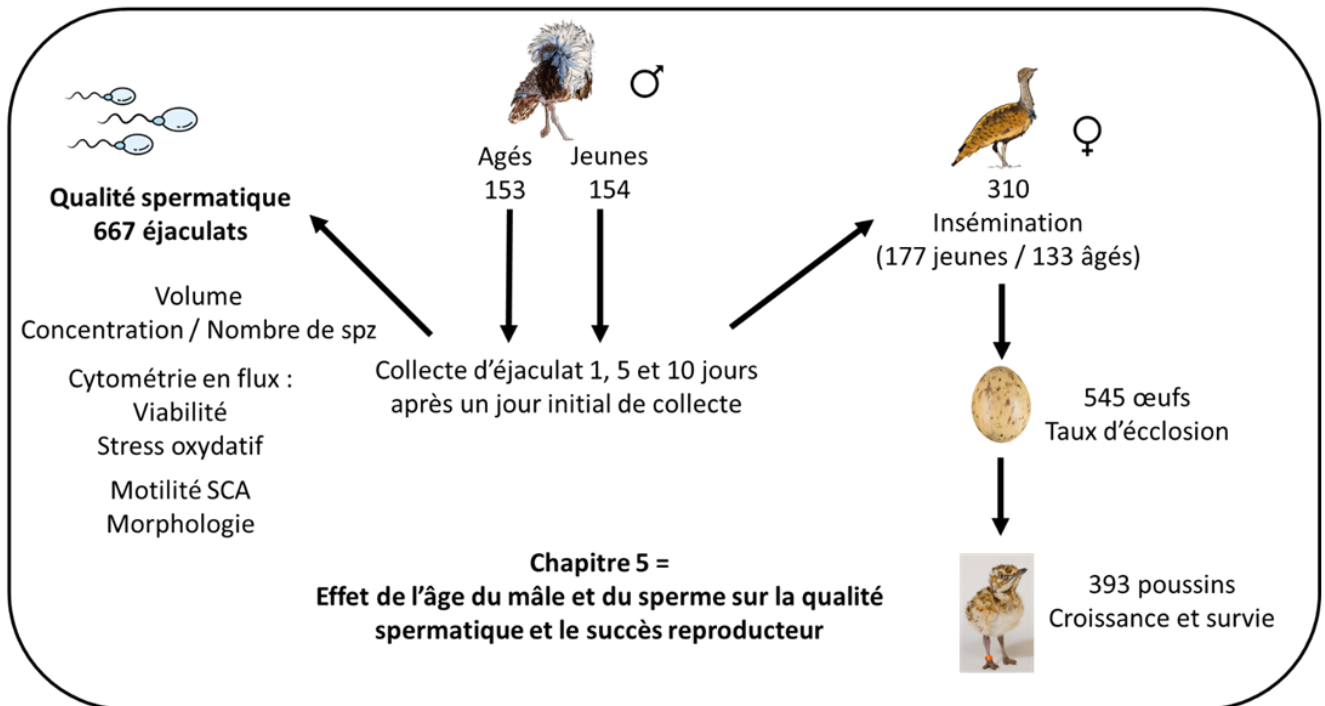


Figure 1 : Synthèse schématique des design expérimentaux mis en place pour les différents chapitres de la thèse. Légendes : spz : spermatozoïdes ; SSTs : tubules de stockage des spermatozoïdes ; LMP : last male precedence

Elevage conservatoire de l'outarde houbara

Tous les oiseaux utilisés dans ce travail de thèse faisaient partie d'un programme d'élevage de conservation à l'Emirates Center for Wildlife Propagation (ECWP) dans l'est du Maroc, les oiseaux ont été répartis dans deux centres dans le département de Fès-Boulemane (localisation GPS (latitude / longitude) : Centre de Missour : (33.007° /-4.098°), et Centre d'Enjil : (33.147° /-4.578°), visant à renforcer les populations naturelles de l'outarde houbara d'Afrique du Nord (Lacroix 2003). Tous les poussins sont produits par insémination artificielle (IA) dans le cadre d'une gestion génétique stricte afin d'assurer une diversité génétique (Rabier et al. 2020 ; Loïc Lesobre 2008). Le flock de reproducteurs compte un total de 7713 oiseaux, 2961 mâles et 4752 femelles, produisant actuellement environ 20 000 poussins par an, utilisés pour la conservation, la translocation et la supplémentation pour la chasse à travers l'aire de répartition de l'espèce. Les reproducteurs sont logés individuellement dans des cages de 4 mètres carrés, tandis que les oiseaux destinés à être relâchés sont gardés dans des volières de 30 mètres sur 9. Plus de détails sur le programme de conservation sont disponibles sur www.houbarafund.org.

Animaux expérimentaux

Tous les oiseaux utilisés pour la collecte de sperme, le prélèvement de sang et l'insémination faisaient partie du flock de reproducteurs. Tous les oiseaux utilisés pour les études anatomiques, histologiques, et de stockage des spermatozoïdes sont nés en captivité et étaient de génération F3 ou plus. Ils ont été considérés comme réformés pour la gestion du troupeau captif ; aucun oiseau qui aurait pu être relâché dans la nature ou conservé comme futur reproducteur n'a été euthanasié. Les reproducteurs sont logés individuellement dans des cages de 4 mètres carrés, tandis que les oiseaux destinés à être relâchés sont gardés dans des volières de 30 mètres sur 9. Tous les oiseaux ont été nourris avec des aliments secs commerciaux spécialement formulés, des vers de farine et des grillons.

Collecte du sperme et analyse de qualité spermatique

Les mâles sont collectés le matin par un technicien qualifié à l'aide d'une femelle factice. Après la présentation, le mâle monte sur le mannequin et commence à copuler. A ce moment, une boîte de Petri en verre est utilisée pour collecter l'éjaculat en la pressant doucement sur le cloaque du mâle pendant l'éjaculation (Fig.15). Le sperme est

immédiatement transféré dans un cryotube de 2 ml et conservé à température ambiante. Les éjaculats sont directement évalués pour la motilité (en utilisant un indice de motilité de masse allant de 0 à 5, voir Vuarin et al. 2019a pour plus de détails), le volume et la concentration en spermatozoïdes (par spectrophotométrie) et dilués (1:1) dans Lake 7.1 (Lake et Ravie, 1984).

Différents paramètres de qualité spermatique ont été analysés. La viabilité, le potentiel mitochondrial, le niveau de stress oxydatif, la fragmentation et la décondensation de l'ADN ont été analysés par cytométrie en flux (Guava easycyte 5HT, MV, L'Aigle, France) et en utilisant différents fluorochromes. La motilité et divers paramètres de vitesse ont été mesurés à l'aide d'un système CASA (SCA, Microoptics, Barcelona, Spain). Pour mesurer la morphologie, 100 spermatozoïdes ont été observés à l'aide d'un microscope à contraste de phase négatif (Nikon E200-LED, Japon; objectif x100, Phase contrast, oil immersion) et classifiés selon 8 catégories : perte de l'acrosome, anomalie de la tête, tête gonflée, anomalie de la pièce intermédiaire, anomalie du flagelle, gouttelette cytoplasmique, anomalie tératogène (double tête et flagelle) et normal.

Insémination artificielle

Pour évaluer le stade reproductif, le développement cloacal des femelles est régulièrement vérifié. L'insémination est effectuée lorsque les femelles sont développées et prêtes à pondre, généralement 3 à 6 jours avant le début de la ponte. Les femelles sont maintenues tête en bas et le cloaque est délicatement ouvert à l'aide de forceps. Le sperme est alors lentement déposé à environ 1 cm à l'intérieur du vagin à l'aide d'un tube d'insémination monté sur une pipette (Fig.16 ; Saint Jalme et al., 1994). Toutes les femelles ont été inséminées le matin avec un volume minimum d'éjaculat de 50µL et un maximum de 130µL. Selon l'expérience, les femelles ont été inséminées avant la première ponte de la saison ou entre la première et la deuxième ponte, sans insémination préalable au cours de la saison et avec ou sans seuils de motilité et de nombre de spermatozoïdes (voir chapitres 5 et 6).

Collecte, incubation des œufs et élevage des poussins

Les œufs ont été collectés quotidiennement, désinfectés et placés en incubation artificielle pendant 21 à 24 jours. Les œufs collectés présentant des défauts tels que des coquilles endommagées ou un poids insuffisant n'ont pas été incubés. L'évaluation de la

fertilité a été effectuée au huitième jour d'incubation par la méthode du mirage. Un œuf a été considéré comme fertile lorsqu'un signe de développement embryonnaire a été observé, tel qu'une chambre à air développée, un embryon visible ou des vaisseaux sanguins. Si aucun signe de fertilité n'a été observé, les œufs ont été ouverts pour distinguer visuellement la mort embryonnaire précoce d'un œuf sans signe de fécondation (clear-ED). La fertilité des œufs a été considérée comme "claire-ED" lorsqu'il n'était pas possible de distinguer les œufs non fertiles de la mort embryonnaire précoce. Les œufs qui n'ont pas éclos après 24 jours d'incubation ont également été ouverts et le développement embryonnaire a été évalué. Lorsque le développement embryonnaire s'est arrêté au cours du deuxième et du dernier tiers de l'incubation, l'œuf a été classé comme étant une mort embryonnaire moyenne (MD) et une mort embryonnaire tardive (LD) respectivement. Les poussins viables éclos ont été élevés et nourris manuellement dans des cages jusqu'à 35 jours, puis transférés dans des volières extérieures.

Collecte de sang, de tissus et d'organes reproducteur

Des échantillons de sang ont été prélevés sur les mâles adultes pour le dosage de la testostérone et sur les reproducteurs mâles et femelles adultes pour l'analyse de paternité. Du sang de poussins et des tissus d'embryons ont été prélevés pour l'analyse de paternité. Les échantillons de sang des adultes ont été prélevés dans la veine jugulaire ou dans la veine brachiale à l'aide d'une seringue de 3 ml et d'une aiguille (23G). Les échantillons de sang des poussins ont été prélevés sur la veine brachiale au jour 1 en utilisant un capillaire de 20 µl sans héparine (Hirschmann, Allemagne) et transférés dans 180 µl d'éthanol absolu. Des échantillons de tissus (muscle, 1mm³) ont été prélevés lors de l'évaluation de la mort embryonnaire. Tous les échantillons ont été stockés au congélateur (-18C) jusqu'à l'analyse.

Pour prélever les organes reproducteurs, l'euthanasie a été pratiquée par un vétérinaire de l'ECWP (1 ml de T61 intraveineux, MSD, 200 mg d'Embutramide, 50 mg d'Iodure de Mebezonium, et 5 mg de Chlorhydrate de Tetracaine, (Baker-Cook et al. 2021)). Au total, 55 adultes sexuellement matures ($N_{\text{mâles}} = 25$, âge moyen mâles \pm SD = $5,16 \pm 0,37$, Min-Max = 5-6 ; $N_{\text{femelles}} = 30$, âge moyen femelles \pm SD = $5,6 \pm 0,72$, Min-Max = 5-7) ont été euthanasiés. La cavité coelomique a été ouverte et les organes reproducteurs retirés. Les organes ont été photographiés, mesurés, fixés en fonction des différentes analyses (histologie ou microscopie électronique, chapitre 1) ou transférés dans du PBS 1X (oviducte pour le chapitre 2).

Déclaration d'éthique

Le programme d'élevage est soumis à l'autorisation du ministère marocain de l'agriculture et d'un vétérinaire indépendant mandaté (numéro de mandat : 534-98), qui assure le contrôle du bien-être des oiseaux. Les installations vétérinaires sur place garantissent des soins adaptés aux oiseaux malades ou blessés par une équipe de vétérinaires experts, et les normes des autorités sanitaires sont régulièrement contrôlées. Les expériences sont menées dans le respect de l'éthique et chaque protocole d'élevage est élaboré en veillant à ce que les oiseaux soient traités de manière appropriée et éthique. Pour minimiser le stress lors de la capture, des chaperons sont utilisés pendant les manipulations. Les installations sont conçues pour assurer le bien-être des oiseaux et pour prévenir les blessures.

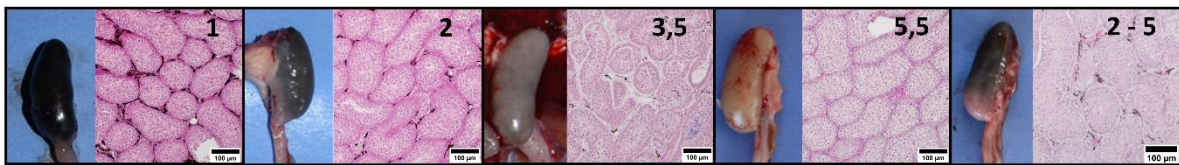
Résultat

Chapitre 1 : Morphologie des spermatozoïdes, description macro et microscopique des tractus reproducteurs mâle et femelle chez l'outarde Houbara d'Afrique du Nord

L'anatomie, histologie des tractus reproducteur mâle et femelle et la morphologie des spermatozoïdes a été décrite pour la première fois chez l'outarde houbara. L'anatomie et l'histologie des organes reproducteurs est similaire à celle d'autres espèces d'oiseaux. Les femelles possèdent un ovaire gauche et un oviducte composé de 6 segments : l'infundibulum, le magnum, l'isthmus, l'utérus, la jonction utérovaginale et le vagin. L'appareil reproducteur mâle est composé de deux testicules, deux épидидymes et deux canaux déférents. Une régression des gonades et des tractus reproducteurs en dehors de la saison de reproduction a été observée. Les mâles possèdent des testicules de couleur sombre, de gris foncé à noir pour la plupart des oiseaux observés. Cette coloration est due à la présence de pigments, en effet des mélanocytes contenant des granules de mélanine ont été observés au microscope optique et électronique à transmission. Ces mélanocytes sont situés dans l'espace interstitiel, entre les tubules séminifères et l'intensité de la coloration des testicules était proportionnelle

à leur abondance (Fig.2). Des granules de mélanine ont également été observés dans le cortex, la médulla et les cellules folliculaires de l'ovaire et au niveau de l'utérus et du vagin.

Figure 2 : Corrélation entre la coloration testiculaire et la quantité de granule de mélanine – Photo anatomique du testicule (à droite) avec le niveau de coloration (de 1 (noir) à 6 (blanc)) et photo microscopique (coloration hematoxyline et eosine - HE, x200) correspondante (à gauche).



Le site stockage des spermatozoïdes a été identifié : des structures similaires aux SSTs d'autres espèces d'oiseaux ont été observées au niveau de la lamina propria de la jonction utérovaginale et de l'infundibulum. La morphologie normale (Fig.3) et les différentes anomalies des spermatozoïdes ont été décrites et quantifiés. Les mâles analysés ($n=166/N_{\text{éjaculats}}=417$) présentés en moyenne 68,5 % de spermatozoïdes normaux, 3,48 % avec une absence d'acrosome, 2,08 % avec une gouttelette cytoplasmique droplet, 3,88 % avec une tête gonflée, 5,35 % avec une anomalie de la tête, 5,35, 8,06 % avec une anomalie de la pièce intermédiaires, 4,57% avec anomalie du flagelle et 4,07% d'anomalie tératogène.

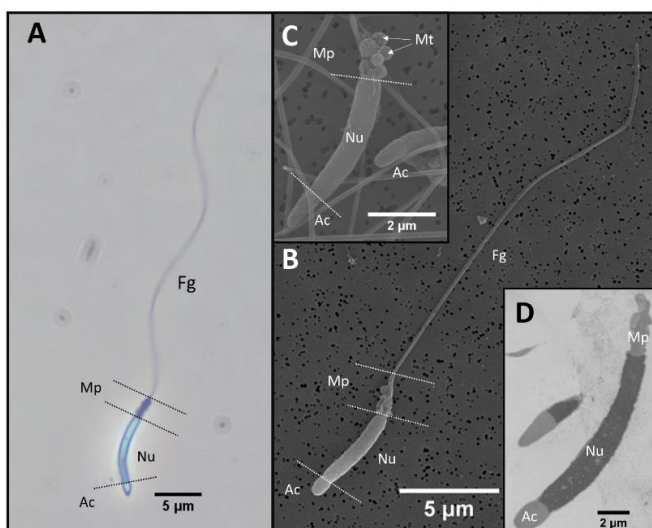


Figure 3 : Morphologie normale des spermatozoïdes d'outarde houbara d'Afrique du Nord observée au microscope à contraste de phase (sous huile à immersion, x1000) (A), au microscope électronique à balayage (B,C) et à transmission (D) – Acrosome (Ac), noyau (Nu), pièce intermédiaire (Mp), mitochondrie (Mt), flagelle (Fg).

Chapitre 2 : Exploration des mécanismes sous-jacent de la LMP chez l'outarde Houbara d'Afrique du Nord

Le stockage des spermatozoïdes dans les SSTs et les différents mécanismes de la LMP ont été étudiés *in vivo* en utilisant une technique de marquage fluorescent des spermatozoïdes. Des femelles ont été inséminées avec des spermatozoïdes marqués par fluorescence (une couleur = un mâle) et les spermatozoïdes stockés de chaque mâle ont été comptés pour un maximum de 100 SSTs. Deux fluorochromes ont été utilisés, un fluorochrome nucléaire bleu Hoechst 33342 (B) et un fluorochrome mitochondrial rouge MitoTracker™ Red FM (MR). Des femelles ont été inséminées avec les spermatozoïdes d'un seul mâle marqué avec le fluorochrome B et ont été euthanasiées à différents intervalles post-insémination (24, 48 et 72 heures) afin de tester le mécanisme de perte passive au cours du temps. Le nombre de spermatozoïdes stockés n'a pas diminué au cours du temps. Des femelles ont été inséminées en séquence avec 48 heures d'intervalle (1^{ère} insémination B / 2^{ème} insémination MR) et euthanasiées 24 heures après la dernière insémination. Les tubules contenaient plus de spermatozoïdes provenant de la deuxième insémination (MR) (Fig.4B/C). De plus, les spermatozoïdes n'étaient pas stratifiés au sein des tubules mais mélangés de manière homogène et les spermatozoïdes de différentes inséminations n'étaient pas ségrégués dans des tubules différents puisque 89% des SSTs observés étaient remplis avec des spermatozoïdes B et MR (Fig.4D). Un déplacement actif des spermatozoïdes semble être le mécanisme principal de la LMP chez cette espèce.

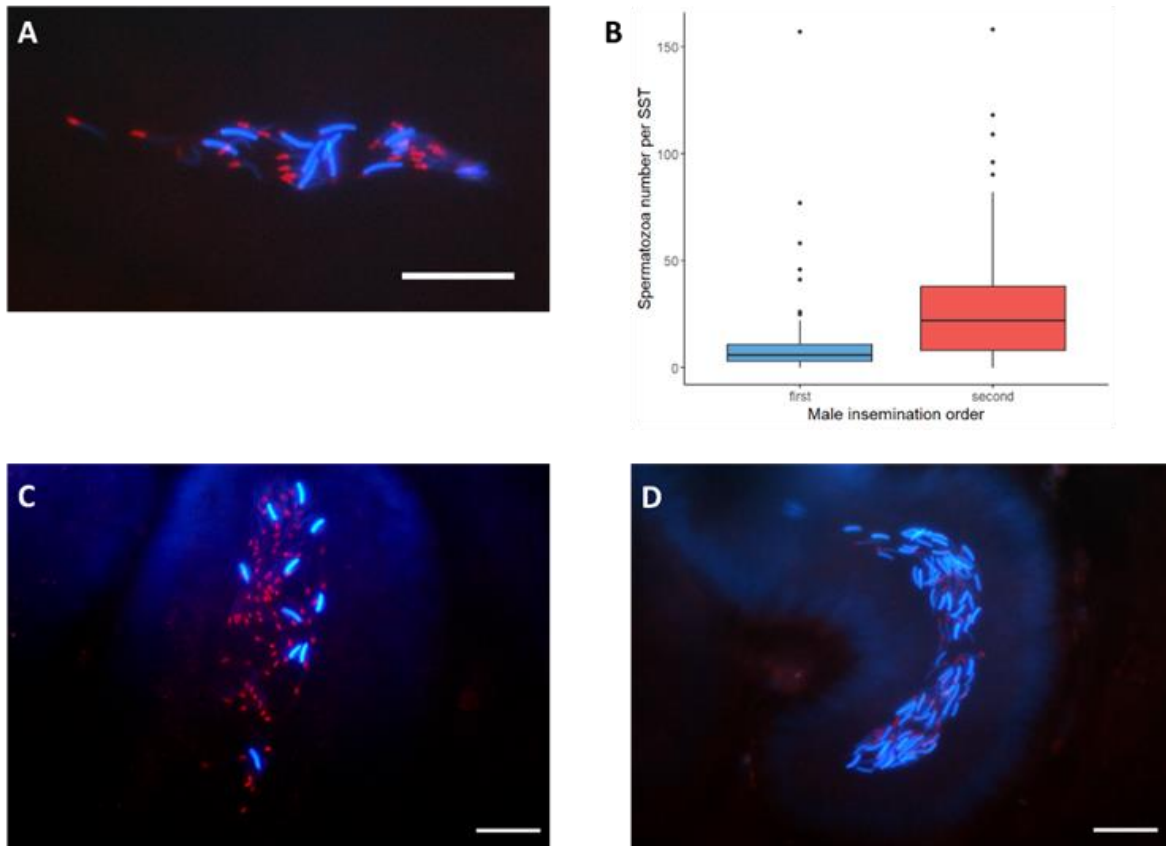


Figure 4: Photo de microscopie à fluorescence d'un SST d'outarde houbara femelle (A) contenant des spermatozoïdes marqués B et MR après insémination avec un mix. Les spermatozoïdes marqués avec B ont un noyau bleu vif alors que les spermatozoïdes marqués avec MR ont une pièce intermédiaire rouge vive et un noyau bleu terne (x400, échelle 20µm) ; (B) Nombre de spermatozoïdes comptés par SST en fonction de l'ordre d'insémination, les valeurs sont exprimés en médiane (ligne noire), premier (boite supérieure) et troisième (boite inférieure) quartiles, minimum (ligne inférieur) et maximum (ligne supérieure) et les points représentés les valeurs aberrantes (N = 2 femelles et 129 SSTs) ; Photo de microscopie d'un SST d'une femelle inséminée avec des spermatozoïdes marqués avec B puis avec MR (C) illustrant le nombre élevé de spermatozoïdes provenant de la deuxième insémination (x400, échelle 20µm) ; et (D) illustrant l'absence de stratification (48 heure intervalle).

Chapitre 3 : L'augmentation de la testostérone précède les pics de parade nuptiale et de qualité spermatique chez des outardes houbara d'Afrique du Nord mâles adultes et âgés captifs

Les niveaux de testostérone ont été mesurés de décembre à juin chez des mâles adultes et âgés. L'âge n'a pas influencé la production de testostérone. Les niveaux ont progressivement augmenté à partir de la première collecte pour atteindre un niveau maximal à la fin du mois de février, puis ont diminué. Ce pic de sécrétion de testostérone a précédé les pics de production et qualité des spermatozoïdes et de fréquence de parade, qui ont été atteints environ 30 jours plus tard, à la fin du mois de mars (Fig.5).

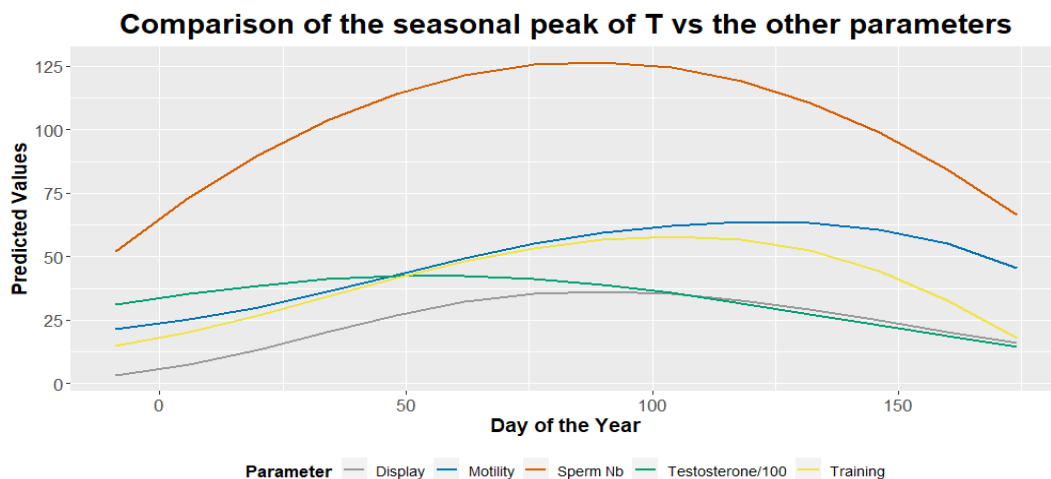


Figure 5 : Tendances saisonnières prévues des niveaux de testostérone, de la probabilité d'accouchement, du nombre et de la motilité des spermatozoïdes, et des performances d'entraînement des outardes houbara mâles. Le 1er janvier a été fixé comme point de référence. Les dates tombant en décembre de l'année précédente sont représentées par des valeurs négatives. Niveau de testostérone (pg/mL*0,01) en vert ; probabilité d'obtenir un score d'entraînement de 5 en jaune (%) ; pourcentage quotidien de mâles en démonstration en gris ; probabilité d'avoir une collection de sperme avec un score de motilité supérieur à 3 en bleu (%) ; (v) et nombre de spermatozoïdes dans l'éjaculat en rouge (*10⁶)

Le pic de testostérone au début de la saison de reproduction permet de préparer la reproduction en intensifiant les comportements de parade et de la production de sperme, puisque la fréquence des parades et le nombre de spermatozoïdes ont augmenté et atteint des valeurs maximales 30 jours plus tard. Bien que les concentrations de testostérone aient

diminué après février, les niveaux sont restés suffisants pour maintenir la production de sperme et l'activité de parade au cours de la saison de reproduction.

Chapitre 4 : Compromis phénotypiques entre la quantité et la qualité des spermatozoïdes en fonction de l'âge chez un oiseau polyandre, l'outarde Houbara d'Afrique du Nord

Lorsque le risque de compétition spermatique est élevé les mâles devraient investir davantage dans la quantité et la qualité des spermatozoïdes. Cependant, en raison des coûts énergétiques ou d'autres contraintes (par exemple, les erreurs de réplication pendant les divisions cellulaires), un compromis entre la quantité et la qualité des spermatozoïdes est attendu. La covariation entre la quantité et la qualité des spermatozoïdes pour différents groupes d'âge (jeune=3-5 ans / vieux=14-20 ans) a été évaluée. Contrairement à ce qui était attendu, il ne semble pas y avoir de compromis entre la quantité et la qualité des spermatozoïdes mais au contraire des covariations positives entre ces paramètres. Les mâles qui produisaient de gros éjaculats (nombre élevé de spermatozoïdes) produisaient également des spermatozoïdes de haute qualité en termes de motilité, de vitesse ou de morphologie normale (Fig.6).

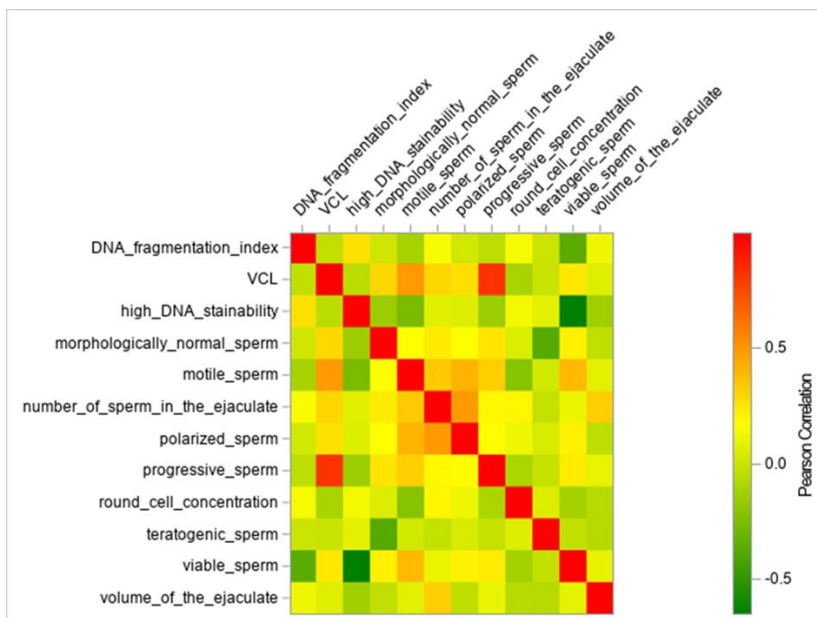


Figure 6. Heatmap illustrant la force et le signe des corrélations entre les 12 caractéristiques incluses dans l'analyse. Le nombre de spermatozoïdes dans l'éjaculat correspond à l'investissement dans la quantité de spermatozoïdes, les 11 autres caractéristiques se rapportant à différents aspects de la qualité de l'éjaculat/du sperme. La taille des échantillons varie de 399 à 454 éjaculats.

De plus, la force de la covariation positive entre la quantité et la qualité des spermatozoïdes ne dépendait pas de l'âge du mâle (pentes similaires entre la quantité et la

qualité des spermatozoïdes chez les mâles en pleine force de l'âge et chez les mâles sénescents). La seule preuve suggérant un compromis entre la quantité et la qualité des spermatozoïdes concerne la relation entre la taille de l'éjaculat et l'indice de fragmentation de l'ADN, puisque les éjaculats contenant un grand nombre de spermatozoïdes présentaient également des proportions élevées de spermatozoïdes dont l'ADN était fragmenté. Globalement ces résultats suggèrent que les mâles qui investissent dans la quantité investissent également dans la qualité de leurs gamètes et donc qu'une coévolution de la quantité et de la qualité a opéré. Il faudrait cependant confirmer ces covariations phénotypiques avec en testant des covariations génétiques.

Chapitre 5 : La sénescence pré-méiotique, mais non post-méiotique, affecte la qualité des spermatozoïdes et le succès reproducteur chez l'outarde Houbara d'Afrique du Nord

Nous avons exploré les potentiels effets additifs ou interactifs de la sénescence des mâles et du vieillissement des spermatozoïdes sur le succès sur la qualité spermatique et le succès reproducteur. Pour évaluer l'effet du vieillissement pré-méiotique, nous avons comparé le succès reproducteur des mâles entre deux classes d'âge (3-6 ans et 12-16 ans). Pour évaluer l'effet du vieillissement post-méiotique, les éjaculats des mâles ont été collectés à trois intervalles de temps après une collecte initiale commune (jour 1, 5 et 10). Par conséquent, les éjaculats du jour 1 sont censés contenir des spermatozoïdes plus jeunes que les éjaculats des jours 5 et 10. Après les inséminations artificielles contrôlées, le succès de la reproduction a été évalué à l'aide de trois traits liés à la condition physique (succès d'éclosion, taux de croissance et de survie des poussins). Outre le rendement reproductif, nous avons également évalué si le vieillissement pré- et post-méiotique affectait différentes caractéristiques des

spermatozoïdes et de l'éjaculat. En accord avec les rapports précédents, nous avons constaté que les mâles âgés produisaient moins de spermatozoïdes avec une motilité plus faible que les jeunes individus. Cependant, contrairement aux prévisions, nous avons constaté que les éjaculats recueillis aux jours 5 et 10 présentaient de meilleures caractéristiques spermatiques, telles que la mobilité et la Vitesse (Fig.7).

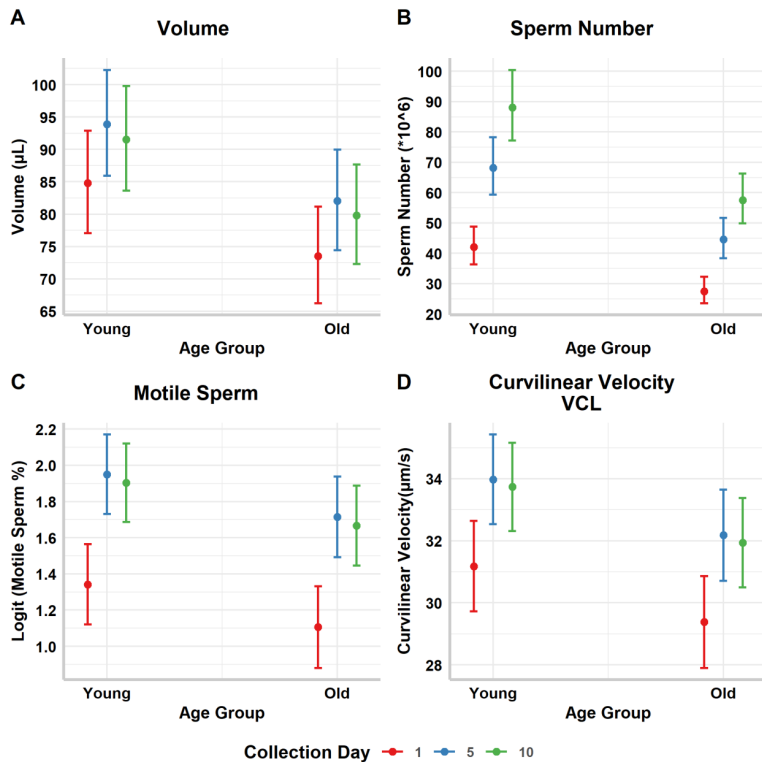


Figure 7 : Moyennes marginales prédites et leurs intervalles de confiance à 95 % correspondants pour (A) le volume de l'éjaculat (μl), (B) le nombre de spermatozoïdes dans l'éjaculat ($\times 10^6$), (C) le pourcentage de spermatozoïdes mobiles (sur l'échelle logit) et (D) la vitesse curviligne, par mâle (vieux vs. jeune) et par âge des spermatozoïdes. Le jour de collecte fait référence au jour où le sperme a été collecté après une collecte initiale commune, et il est utilisé comme indicateur de l'âge des spermatozoïdes.

Les résultats concernant les caractéristiques des spermatozoïdes se sont reflétés sur le succès de la reproduction, puisque les jeunes mâles ont produit des poussins avec une croissance plus élevée et une meilleure survie au cours du premier mois de vie, et que les œufs fécondés par des spermatozoïdes collectés au jour 5 ont le plus grand succès d'éclosion. Aucun effet interactif de l'âge du mâle et des spermatozoïdes n'a été mis en évidence. Dans l'ensemble, ces résultats confirment l'effet de l'âge du mâle sur le succès reproducteur et suggère un processus de maturation dans le tractus reproducteur mâle qui pourrait refléter la fréquence d'accouplement des mâles dans la nature chez cette espèce.

Chapitre 6 : Démêler l'importance des caractéristiques de l'éjaculat, de l'âge et de l'ordre d'accouplement chez une espèce ayant un effet de LMP

Nous avons utilisé une approche expérimentale pour démêler l'importance des caractéristiques spermatiques, de l'âge du mâle et de l'ordre d'accouplement lors d'inséminations compétitives chez l'outarde Houbara d'Afrique du Nord, une espèce d'oiseau dont le système d'accouplement est basé sur les leks. À cette fin, nous avons inséminé artificiellement des femelles à deux reprises avec du sperme prélevé sur des mâles adultes à maturité sexuelle (3-5 ans) et en âge de sénescence (14-20 ans), avec un intervalle de 48 heures entre les deux inséminations. Nous avons également évalué la qualité spermatique des mâles en compétition en utilisant différents paramètres. Nous avons constaté que le sperme utilisé pour les inséminations ayant lieu 48 heures après la première insémination avait un succès de fécondation plus élevé (60%), indépendamment de l'âge du mâle et de la qualité du sperme (Fig.8). Ce résultat montre donc que l'ordre d'accouplement joue un rôle clé dans le succès de fécondation lors d'insémination compétitive et confirme que la LMP opère chez cette espèce.

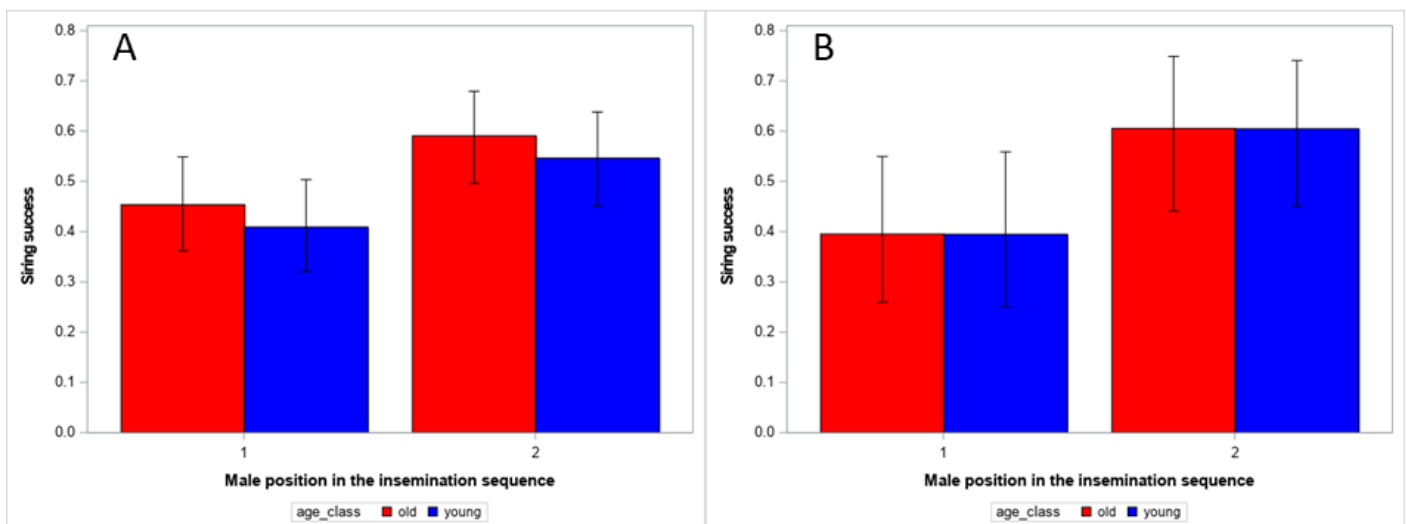


Figure 8. Succès de reproduction des mâles d'outarde houbara d'Afrique du Nord en fonction de leur position dans la séquence d'insémination (1 = premier, 2 = dernier) et de leur âge (jeune = tranche d'âge du pic de fertilité, vieux = sénéscent). Nous rapportons les moyennes des moindres carrés avec les intervalles de confiance à 95%. A) Succès de reproduction incluant tous les œufs pondus (N = 115 mâles et 436 descendants) ; B) Succès de

reproduction basé uniquement sur les premiers œufs pondus (N = 100 mâles et 162 descendants).

Nous avons ensuite étudié dans quelle mesure la différence de qualité des spermatozoïdes en compétition pouvait compenser la LMP. Nous avons constaté que les éjaculats utilisés pour les premières inséminations ne l'emportaient sur les éjaculats utilisés pour les dernières inséminations que lorsqu'ils étaient de meilleure qualité en termes de motilité, de vitesse ou de morphologie normale. Nous n'avons pas trouvé de preuves qui montreraient que les spermatozoïdes de mâles jeunes auraient un avantage compétitif sur les spermatozoïdes des mâles sénescents. Bien que ces résultats soulignent l'importance de l'ordre d'accouplement et des caractéristiques des spermatozoïdes en tant que moteurs du succès reproductif des mâles, ils montrent également que les mâles de qualité relativement inférieure peuvent tout de même réussir à féconder des œufs s'ils parviennent à s'assurer la dernière position dans la séquence d'accouplement.

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