

Sperm-age effects and their biological significance for reproductive success

Nicola L. Hemmings

It has been hypothesised that young sperm may have an advantage over old sperm in terms of reproductive success. Such hypotheses rely on the assumptions that (a) sperm deteriorate with age, and (b) in terms of a female's normal period of sperm use, this deterioration is biologically important. Here it is shown that sperm do deteriorate over time when stored *in vivo* in the female reproductive tract. Limited evidence suggests that some overlap of age-related sperm deterioration and sperm use by females may occur under normal circumstances; however this evidence relies heavily upon a number of important assumptions which reduce its validity. Further, studies of sperm-age effects are subject to a number of confounding factors. A protocol is suggested for future study, with the aim of reducing the constraints and complications imposed by these effects.

Introduction

Since the recognition by Parker [1] that intra-sexual competition between males may continue post-copulation via the process termed sperm competition, much effort has been dedicated to establishing why certain sperm achieve greater reproductive success than others (reviewed in [2]). Factors such as differences in sperm numbers and timing of insemination, sperm form and function, and genetic compatibility with the female have all proved important [3-4]. Less attention, however, has been focussed on the idea that the age of sperm may affect its reproductive potential. This is surprising for two reasons. Firstly, there is a body of evidence suggesting a relationship between sperm age and reproductive success. In *Drosophila melanogaster*, for example, the occurrence of sex-linked lethals in offspring is associated with fertilisation by senesced sperm [5]. Similarly, the use of old sperm has been repeatedly linked to reduced fertility and embryo survival in avian species such as the domestic chicken (*Gallus gallus v. domesticus*) and mallard duck (*Anas platyrhynchos*) (e.g.[6-7]). Mammalian sperm subjected to time in storage (and therefore aged) have also been shown to have reduced fertilisation success, due to a reduction in motility and ability to undergo the acrosome reaction relative to fresher, younger sperm [8-9]. Secondly, it is common among most taxa for sperm to be stored for some duration of time before use, due to delays in reproduction [10] (see Table 1). This means that the age-structure of sperm in the female reproductive tract at any point may be varied and old sperm may have the potential to achieve fertilisation.

The 'young sperm hypothesis' [11] proposes that young spermatozoa have an advantage over old sperm in terms of reproductive success. This advantage can be divided into the following three subsets: 1) greater fertilisation ability; 2) greater zygote survival/hatchability; and 3) greater competitive ability [9-10]. There is circumstantial evidence for all these potential advantages of young sperm. For example, sperm aged in the female reproductive tract has been shown to have reduced fertilising capacity compared to younger sperm in guinea pigs [8] and hamsters [12]; in these cases the ability of sperm to undergo the acrosome reaction appears to become inhibited with age. Black-legged kittiwake (*Rissa tridactyla*) eggs fertilised by old sperm reportedly suffer reduced hatchability [13], and it has been shown in the house cricket (*Acheta domesticus*) that young sperm are more likely to be stored by females following copulation [14], giving young sperm an advantage in sperm competition. Due to the potential benefits of young sperm, using old sperm may significantly reduce a female's reproductive success. Accordingly, sperm age and the need for fresh sperm have been hypothesised as potential reasons for female promiscuity [11]. However, hypotheses suggesting that old sperm may cause problems under normal patterns of reproduction (i.e. where reproductive behaviour is typical for the species), rely on two important assumptions: (a) sperm stored *in vivo* in the female reproductive tract deteriorate over time, and (b) this deterioration coincides with the female's normal sperm storage duration. The problem with assumption (a) is relatively clear; if sperm do not deteriorate to a significant degree with age when stored *in vivo*, old sperm are not likely to have adverse effects on fertility or embryonic survival. However, it seems intuitively obvious that sperm quality should depreciate to some extent with age; most other cells degenerate over time [15], and as we have seen, there is circumstantial evidence that young sperm are more successful than old sperm, supporting the suggestion of a discrepancy between their relative quality. Further, as we will see in the following section, the female reproductive tract provides a relatively hostile environment for spermatozoa (e.g. [16]); it is therefore unlikely that sperm are completely protected from the effects of aging by *in vivo* storage. Assumption (b) is perhaps more critical; age-related sperm deterioration will not be of biological significance to a female if it occurs outside her normal sperm storage period i.e. if she only uses sperm younger than the age at which

Table 1. Reported ranges of maximum sperm storage duration (SSD) for various taxa.

Taxa	Range of S.S.D. across species	Source
Birds	8-117 days	<i>Birkhead and Møller 1993</i> [17]
Reptiles	7-2555 days	<i>Birkhead and Møller 1993</i> [17]
Mammals		
- Excluding bats	0.5-30 days	<i>Birkhead and Møller 1993</i> [17]
- Bats	16-198 days	<i>Birkhead and Møller 1993</i> <i>Racey 1979</i> [17-18]
Fish	Up to 175 days	<i>Darling et al. 1980</i> [19]
Insects		
- Hymenoptera	> 10 years	<i>Taber and Blum 1960</i> [20]

problems begin to occur. However, if sperm reach a problematic age within the functional sperm storage period, age-related sperm deterioration may affect reproductive success and therefore be biologically important.

Despite its ultimate importance for understanding the relationship between sperm age and reproductive success, the biological significance of age-related sperm deterioration does not appear to have been given much empirical consideration. The purpose of this paper is therefore to: (i) review the evidence that sperm stored *in vivo* deteriorate with time; (ii) assess whether this deterioration is biologically significant in terms of normal patterns of sperm use; and (iii) highlight factors that may constrain or complicate testing of sperm-age effects on reproductive success, and suggest a protocol for future studies.

Limitations

The three environments in which sperm awaiting transport to the site of fertilisation may typically be stored are (1) the male reproductive tract (*in vivo*) (e.g. if the male has undergone a period of abstinence); (2) the female reproductive tract (*in vivo*) following insemination (which may or may not involve the use of specialised storage organs depending on the species); and (3) *in vitro* where reproduction is artificially assisted [9].

As the aim of this paper is to elucidate the relationship between sperm deterioration *in vivo* and normal patterns of sperm storage/use by females, focus will be placed on sperm storage occurring *in vivo*, in the female reproductive tract post-insemination. However, it is important to bear in mind the potential importance of sperm storage in the male reproductive tract. An assumption that freshly inseminated sperm equates to freshly produced sperm may be erroneous, potentially leading to an underestimate of sperm age in many studies. To test this assumption, information on rates of spermatogenesis and extent of

sperm deterioration in the male reproductive tract are additionally required. This is beyond the scope of the present paper; however, it may be an important factor and therefore deserves further attention elsewhere.

Consideration should also be given to studies of *in vitro* sperm aging. Although these studies are likely to differ markedly in their results from those of *in vivo* sperm aging, mechanisms of sperm deterioration may follow similar patterns, particularly where attempts are made to replicate the chemical environment of the female reproductive tract *in vitro* (e.g. [21]). Such studies may therefore provide an insight into *in vivo* sperm deterioration. For this reason, when discussing sperm deterioration over time, a number of studies on sperm deterioration over time *in vitro* as well as *in vivo* will be referred to.

When considering the biological significance of age-related sperm effects later in the paper, consideration will also be limited to a single taxon: birds. Birds are particularly suited to the study of these factors for two reasons. First, they have the capacity for prolonged storage of functional spermatozoa within the female reproductive tract. Sperm stored by female birds can remain viable for prolonged periods of time [22] (see Table 1.), often assisted by specialised storage structures [23]. Maximum reported sperm storage durations across species, for example, range from 8-117 days (for comparison mean sperm storage durations of bird species range are reported to range from 6-45 days [24]; see the penultimate section for issues regarding the type of sperm storage duration data used). The domestic turkey (*Meleagis gallopavo*) achieves the record [17], perhaps unsurprisingly considering the high artificial selection pressure placed upon the species due to its commercial importance for the poultry industry. However, several non-domestic species also appear capable of prolonged female sperm storage e.g. the grey-faced petrel *Pterodroma macroptera* (maximum 60 days; [25]) and Buller's shearwater *Puffinus bulleri* (maximum 30 days; [26]). The second reason for focussing on birds is the relative abundance of information available regarding their reproductive behaviour. For many other taxa, particularly vertebrates, there is comparatively little information available (but see [17]).

Sperm Deterioration with Age

Spermatozoa, if not used immediately, are subject to aging just like any other type of cell. Because of the potential periods of sperm storage observed in many animals (see previous section), it is possible that sperm arriving at the site of fertilisation and even fertilising the ovum will have undergone a degree of aging. The current section discusses the potential mechanisms by which sperm of various taxa may degenerate over time. This provides a physiological basis for the following section, where the evidence that old sperm may cause problems for

reproduction is explored (for more comprehensive reviews on factors affecting sperm deterioration see [9; 21; 27]).

Although it seems inherently obvious that young cells should function 'better' than aging cells (just as young individuals are, in general, fitter than old individuals), it is not entirely clear what the cause of any deleterious effects of old sperm may be. Researchers tend to agree that problems most likely arise due to changes in the nuclear material and/or plasma membrane of aging spermatozoa [9; 27], but multiple possibilities remain for what these changes may be and consequently what problems they may cause.

Studies indicate that gross quantitative changes in DNA with sperm aging do not occur, i.e. old or even dead sperm do not appear to have a significantly lower quantity of nuclear DNA than relatively young sperm [28-29]. It is more likely that qualitative changes in the genetic material, such as changes in base-sequence or transcription/translation ability, take place [27; 30]. This suggestion is in agreement with studies on *Drosophila*, where sperm senescence has been shown to have mutagenic effects on progeny [5].

Salisbury and Hart [27] suggest that sperm aging may induce an impairment of RNA translation or genome transcription post-fertilisation. In a study of frogs (*Rana pipiens*), they found that fertilisation by sperm aged *in vitro* resulted in a significant proportion of eggs arrested at gastrulation. The DNA of these arrested gastrulae was identical in quantity, melting point, and priming ability to that of eggs fertilised by un-aged sperm (control). However, when RNA from control gastrulae was injected into eggs fertilised by aged sperm, the result was suppression of gastrula arrest, an effect not seen in a control treatment (injection of RNA from arrested gastrulae) [31]. These results indicate that, in this case, RNA specifically, rather than DNA, was affected by sperm senescence. Furthermore, the arrested gastrulae were shown to have an alteration in their translational mechanism, and also lacked two distinct proteins found in the normal gastrulae, possibly as a result of this.

There are several ways in which senescence may produce negative effects on the physiological make-up, and consequently viability, of spermatozoa. For example, aging may result in increased metabolic instability and a greater rate of metabolic turnover of DNA in sperm. The use of radioactive labelling has shown that, in both sea urchins [32] and bulls [33], sperm may incorporate different bases into their DNA, a process which may lead to new or even nonsense genetic coding and potentially alter protein synthesis [27]. Another possible problem may be the suppression of transcription during embryogenesis due to an increase in protein-binding to DNA in aging sperm [27; 34].

The rate of thermodynamic damage to the nuclear material contained in sperm may also increase with sperm age, both *in vitro* and *in vivo*. Depurination, deamination, and production of thymine dimers occur in the DNA of all

cells, regardless of age [11; 15], but such deleterious effects may accumulate in sperm as they age, due to the relative inability of their DNA to repair itself (a result of the low quantity of cytoplasm possessed by spermatozoa). This damage to sperm DNA may affect not only its fertilising potential, but also its ability to transcribe post-fertilisation. Transcription is essential for zygote formation due to its role in protein synthesis; disrupted transcription may result in development proceeding abnormally.

A popularly hypothesised mechanism for age-related degradation, not only of gametic cells, but also of any living cell, is the effect of free-radical mediated oxidative stress/damage (for a review see [9]). Due to sperm's relative lack of repair mechanisms, aging spermatozoa have the potential for accumulation of damage over time, and may therefore be particularly susceptible to the harmful effects of oxidative damage. Potential results of oxidative damage to spermatozoa may include infertility, problems during embryo development, embryo death, or mortality in offspring's later life [27]. There are multiple different sources and consequences of this process for the biochemical and molecular composition of aging spermatozoa, the most important of which are now briefly discussed.

Firstly, lipid peroxidation appears to be an important degenerative process affecting sperm stored over time [21; 35]. Peroxidation of the highly unsaturated fatty acids, which are an integral component of sperm plasma membranes, increases with sperm storage duration both *in vitro* and *in vivo* [21; 36], compromising membrane integrity. Lipid peroxidation can reduce the ability of sperm to undergo the acrosome reaction and is correlated with male infertility [37]. It is possible that lipid peroxidation of sperm plasma membranes may also interfere with female sperm selection [16], particularly if mechanisms of sperm selection depend on some form of information being expressed on the sperm surface (e.g. [38]).

Second, oxidative stress is also associated with damage to the condensed nuclear material within the spermatozoan [35]. Sperm aged *in vitro* have been reported to suffer from increased base oxidation and denaturation of DNA [39-40], chromatin decondensation [41], and, as was mentioned above, alterations in both DNA-protein complexes (due to increased protein-binding) and base sequences [27].

Third, any mechanism by which the effects of antioxidants are suppressed is likely to lead to increased oxidative stress [9; 21]. It is known that naturally produced antioxidants act to reduce the harmful effects of oxidative stress on sperm stored *in vivo* [36-37], and this knowledge has been applied to *in vitro* storage techniques [21]. Increased sperm storage duration may be linked with a reduction of antioxidant concentration or a suppression of antioxidant activity (e.g. [42]), resulting in a greater extent of oxidative damage occurring in older sperm.

Finally, the endometrial cells of the female reproductive tract produce, among other chemicals, the reactive oxygen species hydrogen peroxide [43]. This provides further oxidative stress for sperm stored *in vivo*. The longer their duration of storage in the female tract, the more exposed spermatozoa will be to this oxidising agent, so old sperm i.e. those stored for long durations will accrue the most damage. It is therefore unlikely that *in vivo* storage acts to protect the viability of sperm as they age; in fact the female reproductive tract is more likely a hostile environment for sperm, intensifying sperm competition [16].

The potential for spermatozoa to degenerate and become less viable as they age, both *in vivo* and *in vitro*, has been clearly shown by experimental study. However, if a particular species does not require stored sperm for fertilisation, e.g. if copulations between a pair are frequent up until fertilisation, the issues associated with fertility of old sperm will be irrelevant. Similarly, if old sperm are relatively infertile, problems with embryo development caused by sperm senescence should also be unimportant, as old sperm will be incapable of fertilisation. In terms of normal fertility and sperm storage, the likelihood that sperm aging effects will be of any biological significance for females will now be discussed.

Biological Significance of Sperm Deterioration

The duration of functional sperm storage in birds depends on the following factors: (i) copulation frequency; (ii) laying interval (i.e. time between successive eggs); and (iii) clutch size. Whether or not age-related deterioration of sperm is a problem for females will depend on the timing of sperm deterioration relative to the functional sperm storage duration. Consider a hypothetical example; a bird lays a mean clutch size of six. She copulates on the day of fertilisation and lays one egg per day. Assuming fertilisation occurs 24hrs before egg-laying (as is typical for many birds) and no further copulations occur during the period of egg-laying, this bird will need to use sperm stored for up to six days. If sperm only begin to deteriorate with age after e.g. eight days, the female will never need to use old sperm and is therefore unlikely to suffer any associated fitness consequences. However, if sperm begin to deteriorate before six days of storage, their potential adverse effects may become an issue.

Data on the timing of sperm deterioration in relation to sperm storage duration is lacking, as is detailed information on copulation frequency for different species. However, relevant data do exist at least for domestic chickens (*Gallus gallus v. domesticus*) and mallard ducks (*Anas platyrhynchos*). In chickens, Lodge *et al.* [6] reported a trend toward increased embryo death with sperm storage duration from the fifth day of egg-laying onwards. The first fertile eggs were collected on the second day after insemination [6], and in chickens, eggs are fertilised 24hrs before they are laid. Problems

associated with increased duration of sperm storage therefore appear to begin after 5 days sperm storage (Fig. 1). Domesticated chickens are the direct descendants of red jungle fowl (*Gallus gallus*); this wild species therefore provide us with relevant information on 'normal' reproductive patterns (i.e. that not influenced by domestication and experimental techniques (see next section)) with which to compare this data. Red jungle fowl lay one egg per day, with a mean clutch size of 5.5 ± 0.5 [44]. Assuming the female is inseminated the day before the first fertile egg is laid and no subsequent insemination occurs throughout the period of egg-laying, sperm will therefore need to be stored for 5.5 ± 0.5 days. The period of sperm use therefore overlaps marginally with the onset of age-related sperm deterioration (Fig 1), although even with a clutch size of six (mean+S.D.) any adverse effect would act only on the last egg.

In mallard ducks (*Anas platyrhynchos*), a marked increase in early embryo mortality (EEM) appears to occur from the ninth day onwards following a single insemination [7]. However, the data from this study [7] are presented in grouped intervals of 2-4, 5-8, and 9-11 days after insemination, making it difficult to accurately determine the precise day on which this effect becomes significant. For example, if a particularly high incidence of EEM occurs on day 11 after insemination, this will raise the mean incidence for the whole interval of 9-11 days, even if days 9 and 10 are not associated with particularly high incidence of EEM. However, if it is assumed that the onset of age-related sperm deterioration does occur from 9 days onwards, it is possible to estimate the extent to which normal sperm storage duration will overlap with the onset of sperm-age related problems. The mean clutch size for wild strains of mallards is 11 ± 2 [45]; if the same assumptions regarding timing of copulations are applied as with the chickens (above), mallard ducks may need to store sperm for 11 ± 2 days, a period which may overlap with the onset of age-related sperm deterioration (assumed to be from 9 days onwards) by up to 4 days. Because sperm deterioration may begin later than assumed here, this overlap may be overestimated. Information on daily EEM is required for a more accurate measure.

From these data it appears that the normal period of sperm storage for both these species may coincide to some degree with the stage at which aging sperm may begin to cause problems. However, the assumptions made regarding timing and frequency of copulations may reduce the reliability of these data. For example, copulations may not always occur the day before the first egg is laid, so functional sperm storage durations may be longer than suggested here. This would lead to a greater use of aged sperm and therefore potentially reduced reproductive success. In contrast, the opposite effect would occur if copulations continued into the egg-laying period (as they are assumed not to here). Sperm would not need to be stored for as long and, as a result, the likelihood of suffering related fitness consequences would be low.

Importantly, chickens do normally copulate the laying period (C. Cornwallis and T. Pizzari, pers. comm.); this clearly reduces the likelihood of old sperm being used, and suggests that, at least for this bird species, age-related sperm deterioration may not be biologically significant. Unfortunately, this information could not be obtained for mallards.

There are many problems associated with the study of sperm storage durations and age-related effects of sperm on reproductive success which may reduce the reliability of results. One example we have already encountered; due to a relative paucity of information regarding specific aspects of reproductive behaviour across species, making assumptions is often unavoidable, and yet such assumptions can lead to inaccuracy. The remainder of this paper will outline, and suggest ways of controlling for, the various other problems and confounding factors associated with this area of study.

Problems for the Study of Sperm-Age Effects

Sperm storage data

Several potential problems for the application of sperm storage data exist, and these must be considered when using or interpreting such data. The first involves the type of sperm storage data which are used i.e. mean, median or maximum values. Table 1. presents maximum reported sperm storage durations for various taxa. Maximum sperm storage durations are arguably the most accessible

data currently available; however, they are generally based on only a single example which may reflect a chance event. Furthermore, published results may be biased towards extreme values [22;46]. Despite evidence of a correlation between maximum, mean and median sperm storage duration in four species of bird [22], maximum values are likely to have limited reliability as an index of sperm storage duration, and should therefore be used with caution. When available, mean values of sperm storage duration are more desirable.

Secondly, most confident reports of prolonged sperm storage are from studies of an experimental nature, and females are therefore unlikely to be subjected to or exhibit normal reproductive patterns. For example, although females may be capable of storing viable sperm for long periods of time when necessary i.e. in the absence of fresh sperm, under normal patterns of copulation they may never need to do this because fresh sperm may be readily available. Studies which permit females only a single insemination and then measure the period of functional sperm storage by examining the resulting consecutive eggs (e.g. [6-7; 47]) do not necessarily demonstrate typical reproductive behaviour of the species studied; in particular, normal sperm storage durations may be overestimated. In order to obtain a more realistic idea of sperm storage duration, studies need to consider other aspects of the species' 'normal' reproductive behaviour (i.e. when not subject to experimental manipulation), such as clutch size

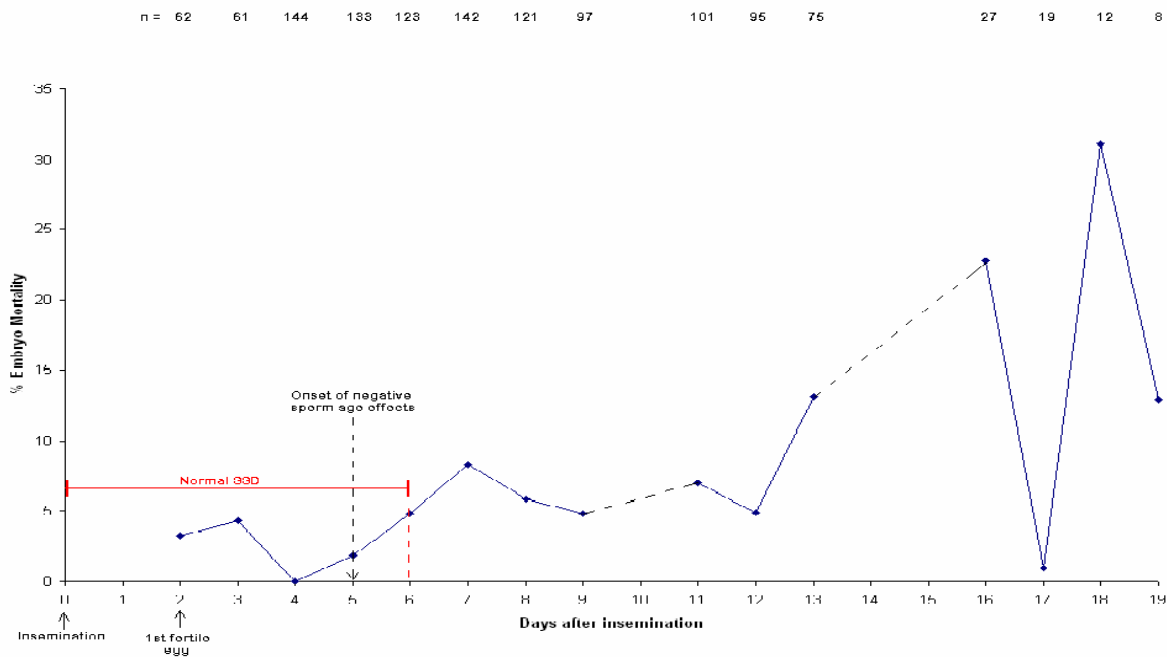


Figure 1. Effect of sperm storage duration on percentage fertile eggs suffering embryo mortality in the chicken, showing the overlap between the onset of sperm-age effects and normal sperm storage duration (SSD) (data extracted from Lodge et al. [6]). Day 0 is the day of insemination; day 2 is the first day of fertile eggs.

and copulation frequency.

Finally, it is possible that fertility may sometimes be confused with parthenogenesis (i.e. the occurrence of cell division in the female gamete without fertilisation, potentially resulting in embryonic development [48]). Occasional parthenogenesis has been reported in various taxa (e.g. reptiles [49]; fish [50]; birds [51]), and it may be difficult to distinguish between development resulting from this phenomena and that resulting from normal fertilisation. Only studies which look directly for sperm associated with the ova can conclusively assert true fertility [52].

Comparability of captive and wild animals

The application of information from experimental studies on factors affecting sperm storage duration, such as clutch size and copulation frequency, to wild species relies on an important assumption; captive populations used in experimental studies are comparable to their wild counterparts. However, it is likely that captive animals will differ from wild animals to some degree; in particular, domesticated species such as the chicken and turkey, which are subject to artificial selection for increased productivity, are likely to exhibit very different reproductive behaviour compared to that of wild strains [53].

Indeed, where clutch size is concerned, a discrepancy in reproductive success does appear to occur between wild and captive birds. Clutch size recorded from experimental study in captivity is greater than that recorded from observation in the wild in 13 of 15 species for which the data are available [22; 44-45; 54-56] (Table 2.), a relationship which approaches significance (paired t-test: $t=1.56$, $d.f.=28$, $p=0.066$). This difference is particularly notable where those birds studied in captivity are domesticated strains of the wild species they are compared with e.g. the domestic and wild turkey (*Meleagris gallopavo*), the domestic duck and mallard duck (*Anas platyrhynchos* and *Anas platyrhynchos var. domesticus* respectively), and the domestic chicken and red jungle fowl (*Gallus gallus var. domesticus* and *Gallus gallus* respectively) (see Table 1.). If birds have smaller clutches in the wild compared to captivity, they will be less likely to use sperm which have aged to a disadvantageous degree. Assuming that the interval between insemination and fertilisation and laying intervals remain the same, smaller clutches will result in sperm being used over a shorter duration of time. Sperm used will therefore tend to be younger.

Artificial insemination

Another difference between captive and wild animals lies in the methods by which their reproductive behaviour is studied. Experimental techniques which force a departure from normal reproduction are likely to yield results which are relatively inapplicable to natural systems. The problems this may have for sperm storage data have been mentioned previously. However, the use of artificial

insemination (A.I.) also creates similar difficulties. For example, in domestic chickens, A.I. commonly results in over 100 million spermatozoa being transferred into the female reproductive tract. In comparison, natural inseminations in red jungle fowl only transfer in the region of 5 million spermatozoa (T.R. Birkhead pers. comm.). The importance of sperm numbers for reproductive success is discussed further in the following sub-section.

Another potential problem exists with the way in which many A.I. techniques transcend post-copulatory pre-zygotic barriers to fertilisation [57]. The result of this may be that unfit sperm, which under normal circumstances may not advance through the female reproductive tract, are able to achieve fertilisation. Fertilisation success may therefore rise, but as a consequence, negative effects on embryo viability may also increase.

Sperm numbers

Many results which are interpreted as evidence for old sperm causing problems (e.g. [6-7]) are confounded by the fact that they do not control or account for the temporal loss of sperm from storage sites. It is known that, at least in the female reproductive tract, the number of stored sperm declines over time [47; 58]. Further, in a study of breeding hens (*Gallus gallus v. domesticus*), Eslick and McDaniel [59] showed that the number of spermatozoa inseminated had a significant effect on fertility and hatchability independent of sperm age. Low numbers of sperm resulted in decreased fertility, decreased hatchability, and increased early embryo mortality and total mortality. These results suggest that observations of decreasing fertility with sperm storage duration (e.g. [6]) may simply reflect a sperm numbers effect (i.e. be independent of any sperm-age effects). Sperm depletion will also limit scope for sperm competition and sperm selection, which both act to increase the quality of sperm fertilising the ovum; it is feasible therefore that lower numbers of sperm may also act to increase rates of embryo mortality independent of sperm age. As a result, observations of reduced embryo survival with increased sperm storage duration may be explained by lower numbers of sperm or volume of semen available at the point of fertilisation rather than decreased sperm viability with age. Perhaps an even more feasible scenario is that the effects of both aging and depletion of sperm coincide, working either additively or synergistically to reduce reproductive success.

Unfortunately, disentangling the relative effects of sperm numbers and sperm age on reproductive success is difficult and does not appear to have thus far been adequately achieved by experimental study.

Distinguishing between true infertility and early embryo mortality

The majority of studies distinguishing between fertility, infertility, and embryo mortality (EM) do so on the basis of either (a) candling eggs at a set time (usually 3-4 days

Table 1. Comparison of captive and wild clutch sizes in birds.

Species	Clutch Size	
	Captive	Wild
African collared dove <i>Streptopelia roseogrisea</i>	2	1.5
American kestrel <i>Falco sparverius</i>	4.6	4.1 ¹
Bengalese finch*/Sharp-tailed munia <i>Lonchura striata</i>	6	4.3
Bobwhite quail <i>Colinus virginus</i>	14	13.5
Budgerigar <i>Melopsittacus undulates</i>	6	5
Red jungle fowl/Domestic chicken* <i>Gallus gallus (v. domesticus*)</i>	12	5.5
Feral pigeon <i>Columba livia</i>	2	2
Greylag goose <i>Anser anser</i>	12	5
Helmeted guineafowl <i>Numida meleagris</i>	14	9
Japanese quail <i>Coturnix japonica</i>	8	6.5
Mallard duck/Domestic duck* <i>Anas platyrhynchos (v. domesticus*)</i>	17	11
Ring collared pheasant <i>Phasianus colchicus</i>	11.8	11.5
Turkey*/Wild turkey <i>Meleagris gallopavo</i>	17	11.5
Willow grouse <i>Lagopus lagopus</i>	7.5	7.5
Zebra finch <i>Taenopygia guttata</i>	6	4.7 ²

Values are means or medians. Captive values are from [22]; wild values are calculated from [44-45; 56] unless otherwise stated (see below).

¹[54]; ²[55]

*Domestic strains of the wild species

after the start of incubation) or (b) macroscopic/stereomicroscopic examination of the germinal disc (GD) and classification according to Eyal-Giladi and Kochav (EGK) [60] (e.g. The latter method is only usually employed after candling, for eggs which are candled as infertile or as having a dead embryo. However, these methods are not adequate for distinguishing between true infertility and cases of early EM (EEM), i.e. where the embryo has died within 48hr of fertilisation, before the development of apparent blood vessels or embryonic tissue. This is due to difficulties in accurately detecting differences between the gross appearance of the GD for these two categories (see [52]).

Such inaccuracy may lead to cases of EEM being missed and therefore an underestimation of the overall incidence of EM [6; 52]. If such cases of EEM are missed in eggs produced early in the laying period, this may mean that the stage at which adverse sperm-age effects are reported to begin may be inaccurate, and sperm may cause problems after a much shorter duration. Alternatively, if EEM is mistaken for infertility in later eggs, the proportion of hatching failure attributed to EM, possibly as a result of age-related sperm deterioration, will be underestimated.

Infertility is shown to markedly increase with sperm storage duration in studies using the techniques described above (e.g. [6-7]); whether a significant proportion of this apparent infertility can actually be attributed to EEM requires more sophisticated methods of examination. The techniques described by Gupta and Bakst [61] allow a high degree of accuracy when distinguishing between true infertility and EEM. Apparently infertile GDs should be extracted from the egg, stained with fluorescent dye, and

examined microscopically to check for the presence of any nuclei. If nuclei are present, it is likely that fertility followed by EEM, as opposed to infertility, has occurred (however, care must be taken to avoid confusing fertility with parthenogenesis (see [52]). Examination of the perivitelline layer (PVL) for sperm and hydrolysed holes which result from sperm undergoing the acrosome reaction will provide further confidence of fertility [58]. The use of a live-dead assay on nuclei from the GD is also desirable in order to avoid confusing dead embryos with those which are alive but have developed very slowly [52].

Protocol

Due to the problems and confounding factors associated with studies of sperm-age effects on reproductive success, future studies should allocate more effort to controlling for such effects. The following four criteria are therefore suggested for use as a protocol to facilitate the unambiguous demonstration of sperm-age effects.

1. *Control for sperm number effects:* Sperm stored in the female reproductive tract are subject to temporal decline in numbers as well as aging. Because it has been shown that low sperm numbers result in reduced reproductive success [59], the effects of declining sperm numbers and differences in insemination concentration therefore need to be controlled. There may be enough natural variation in sperm numbers from different inseminations to allow the effects of sperm numbers and age to be disentangled without using artificial techniques. This would be possible if, for example, the incidence of EM (a) found in early eggs (i.e. those produced at the start of the laying period)

laid by a female inseminated with a low number of sperm was compared to the incidence of EM (b) found in late eggs (i.e. those produced late in the laying period) produced by females inseminated once with a relatively high number of sperm. The initially high number of sperm will decline by the time these late eggs are fertilised to a number comparable to that in (a). If EM is equivalent for both 'treatments', sperm number effects can be inferred. If EM is higher in late eggs than early eggs despite the comparable sperm numbers, sperm age effects are of importance. Sperm numbers inseminated can be estimated from the number trapped in the perivitelline layers of avian eggs [58].

2. *Clearly distinguish between true fertility and EEM:* To ensure the potential effects of sperm age on EM are not underestimated, a high degree of accuracy is required when classifying eggs as truly infertile. The techniques for examining eggs described above and elsewhere [52; 61] should be used to certify classification of apparently infertile eggs.

3. *Minimise problems associated with sperm storage data:* Studies should obtain mean sperm storage data for the particular species under consideration, and where possible, from large sample sizes. The possibility of parthenogenesis also needs to be excluded; the same techniques for examining eggs as mentioned above can be used to detect parthenogenetic development (see [61]).

4. *Control for differences between wild and captive animals:* This is probably the most difficult criteria to fulfil. Only if accurate information for every relevant aspect of a particular species' normal reproductive behaviour is available will it be possible to apply theory from studies of captive systems to wild systems. This will involve much time and effort dedicated to observational study of wild species. However, a good indication and reference point for the extent to which wild and captive systems differ may be provided by detailed comparisons between domestic and wild strains of species, e.g. the domestic chicken and red jungle fowl (*Gallus gallus v. domesticus* and *Gallus gallus* respectively) and the wild and domestic turkey (*Meleagris gallopavo*).

Conclusion

As was highlighted at the beginning of this paper, hypotheses suggesting that sperm age may affect reproductive success rely upon two assumptions; that (a) sperm deteriorate with time stored in the female reproductive tract, and (b) in terms of normal patterns of sperm use, this deterioration is biologically significant.

The potential for old sperm to cause problems for reproduction is clear. As we have seen, there are numerous mechanisms by which sperm stored in the female reproductive tract may deteriorate over time [27], and this deterioration has been shown to decrease fertilising ability, increase embryo mortality, and lead to

health problems in offspring later life [9-10]. Assumption (a) can therefore be considered justified.

Assumption (b) is more problematic. For age-related sperm deterioration to be of biological significance, its onset must coincide with the normal period of sperm storage in females, which in turn depends on a number of specific aspects of reproductive behaviour. The limited evidence presented here suggests that the timing of sperm-age related problems may overlap to some degree with normal patterns of sperm use. However, these findings rely heavily on some important assumptions, reducing their reliability. Consequently, the validity of assumption (b) cannot yet be demonstrated.

Studies of female sperm storage and the effects of sperm age on reproductive success are generally constrained and complicated by a number of confounding factors, as outlined earlier in the paper. The protocol suggested here for future studies aims to control for these factors, disentangling sperm-age effects from a mesh of other possibilities, and increasing the reliability of results. This, combined with an effort to enhance current knowledge of specific aspects of reproductive behaviour, will result in a greater insight into the biological significance of age-related sperm deterioration. Resulting information may have potentially great implications for our understanding of reproductive behaviour, as well importance for conservation and modern fertility techniques.

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Sperm Age and Reproductive Success

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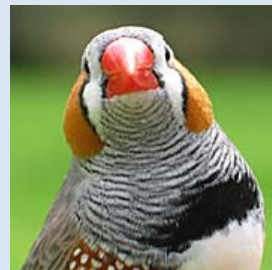
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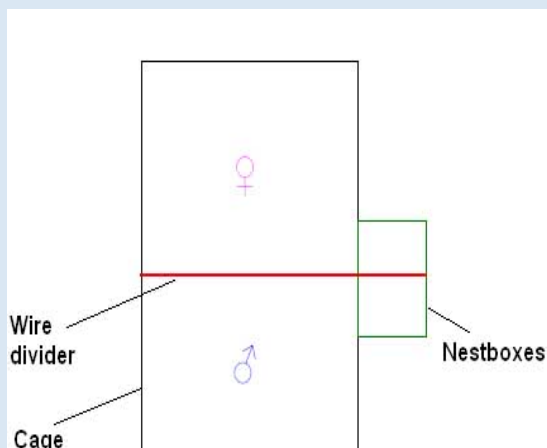
The University Of Sheffield.

Basic methods

The zebra finches (*Taeniopygia guttata*, pictured right) used for this study are from a domesticated population maintained at the University of Sheffield since 1985. Experimental birds are subject to a 12h photoperiod and provided with food *ad libitum*. Their diet is also supplemented with hard-boiled egg to encourage egg production.



Each male and female pair occupy a double cage (each single cage measuring 0.6x0.5x0.4m) with a wire divider down the middle (see diagram below left). Both birds are kept together on one side of the divider until the first egg is laid, after which the male is moved to the other side. The function of the wire divider is to prevent further insemination whilst allowing the pair to remain 'together', encouraging the female to lay normally. In addition, a number of pairs were left together in a single cage to lay normal clutches, whose eggs were used to see if an index of developmental stage could be established from nuclei numbers in the GD (see below).



Eggs are usually laid during the early daylight hours, so nestboxes are checked early in the morning and any eggs are removed and either placed directly into warm incubation or rapidly cooled and stored to be incubated at a later stage. Nestboxes are also checked in the early afternoon for any eggs which have been laid later stage. The first egg laid is replaced with a 'dummy' egg; subsequent eggs are not replaced to encourage the females to continue laying, achieving super-normal clutch sizes.

Eggs are subjected to a set period of warm incubation, the duration of this period depending on what they are being used for. Initially, all eggs were been incubated for 24h. After some preliminary work, this standard incubation period has been changed to 53h (see [Preliminary Results and Discussion](#) for the reasons behind this). Of the eggs from those pairs left together to lay normally, the first and third are incubated for around 4 days then candled. This allows some confidence that the eggs are developing normally. Of those clutches where both candled eggs are fertile and developing normally, another 4 eggs are examined, after incubation periods of either 0h, 24h, or 48h. The rationale behind this is that nuclei number may be able to provide an index for developmental stage. After the 6th egg no more are used for this purpose to minimise the chance of EEM interfering with results.

Due to time constraints most eggs were subjected to some period of cool incubation/storage; however, total cool storage prior to warm incubation never extended beyond 96h.

Techniques for distinguishing fertility and infertility

Please refer to the figures below which depict different stages of the examination process.

- On initial break-out of the egg, the gross appearance of the germinal disc (GD) is examined and photographed. Figure 1 shows the GD of a fertile egg examined after 24h incubation, with the area opaca (outer ring) and area pellucida (pale inner area) clearly visible. Figure 2 shows a fertile GD after 53h incubation; development has clearly proceeded, with the GD becoming 'pear-shaped' and the primitive streak beginning to form (appears initially as a pale line down the centre of the area pellucida). There is a clear difference between the gross appearance of these fertile GDs and the infertile GD shown in Figure 3, which appears as a relatively dense whitish spot. However, these differences are not always so clear, particularly if early embryo mortality (EEM) has occurred before the area opaca and area pellucida become clear.
- Once the gross appearance has been noted, the GD is removed from the egg as follows to allow microscopic examination. A ring of filter paper is placed on the yolk, framing the GD (Figure 4). The perivitelline layer (PVL) adheres to this filter paper allowing the PVL above the GD to be cut out (using iris scissors) and lifted from the yolk, taking the GD with it.
- The excess yolk is then cleaned away from PVL and GD while they are still mounted on the filter paper, using a fine hair loop and gently washing in PBS (Figure 5).
- The GD is then completely lifted away from the PVL (Figure 6) to be examined for nuclei. The GD matter is dispersed in PBS by gentle flushing in and out of a pipette. Half of this solution is stained with a fluorescent DNA dye Hoechst 33324 and examined for nuclei under a fluorescence microscope with a BP 340-380 excitation filter and a LP 425 suppression filter. Nuclei will stain bright blue (Figure 7). Nuclei numbers are estimated by counting numbers in several fields of known area and extrapolating. These estimates will be used to see if an index of developmental stage can be deduced from nuclei numbers. The other half of the GD solution is treated with a live-dead assay consisting of Propidium Iodide and SYBR-14 to assess the proportion of live cells in the GD. However, there are a number of complications involved with the use of this assay which will be described in the [Preliminary Results and Discussion](#) page.
- The PVL is also examined to find and count sperm and sperm holes. First, the section of PVL from above the GD is peeled away from the filter paper ring. The PVL consists of both inner and outer membranes; sperm make holes in the inner PVL when undergoing the acrosome reaction to enter the ovum. Once this has occurred the outer PVL is laid down, trapping sperm present at the time of fertilisation. These two layers can be separated by gentle agitation in PBS and be examined separately for sperm and sperm holes.

- The outer PVL (both from above the GD and the rest of the membrane) is laid out on a microscope slide, stained with Hoechst and viewed in the same way as the GD nuclei. Any sperm stain bright blue (Figure 8) and are counted systematically by scanning the slide.
- The inner PVL is also laid out on a microscope slide and viewed under a light microscope with dark field optics to view any holes created by sperm undergoing the acrosome reaction (Figure 9).



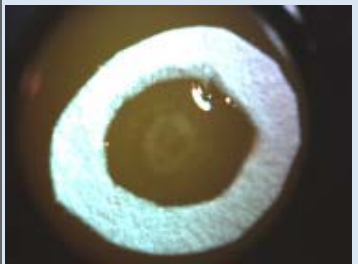
1: Fertile GD after 24h incubation



2: Fertile GD after 53h incubation



3: Infertile GD (note its dense white spot-like appearance)



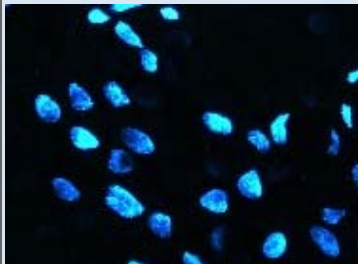
4: Yolk with filter paper framing GD



5: GD framed with filter paper removed from yolk and cleaned



6: GD completely separated from yolk and PVL



7: Nuclei from GD stained with Hoechst



8: Spermatozoa on outer PVL stained with Hoechst



9: Holes made by sperm in inner PVL