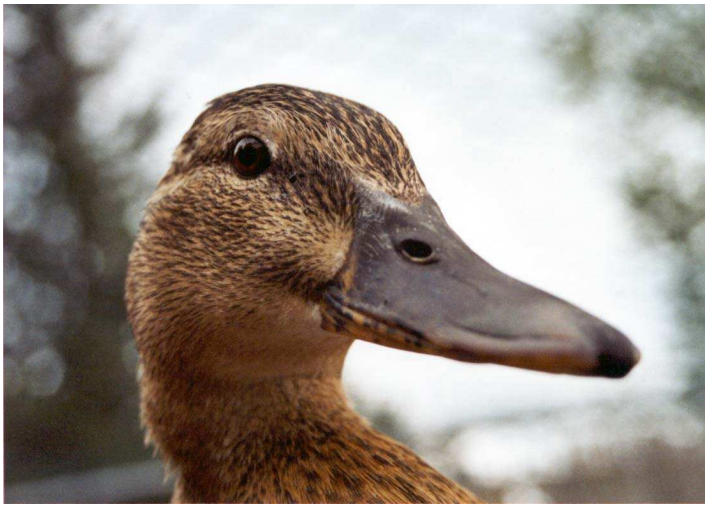
MALE AND FEMALE REPRODUCTIVE TACTICS IN MALLARDS (*ANAS PLATYRHYNCHOS* L.): SPERM COMPETITION AND CRYPTIC FEMALE CHOICE



DISSERTATION

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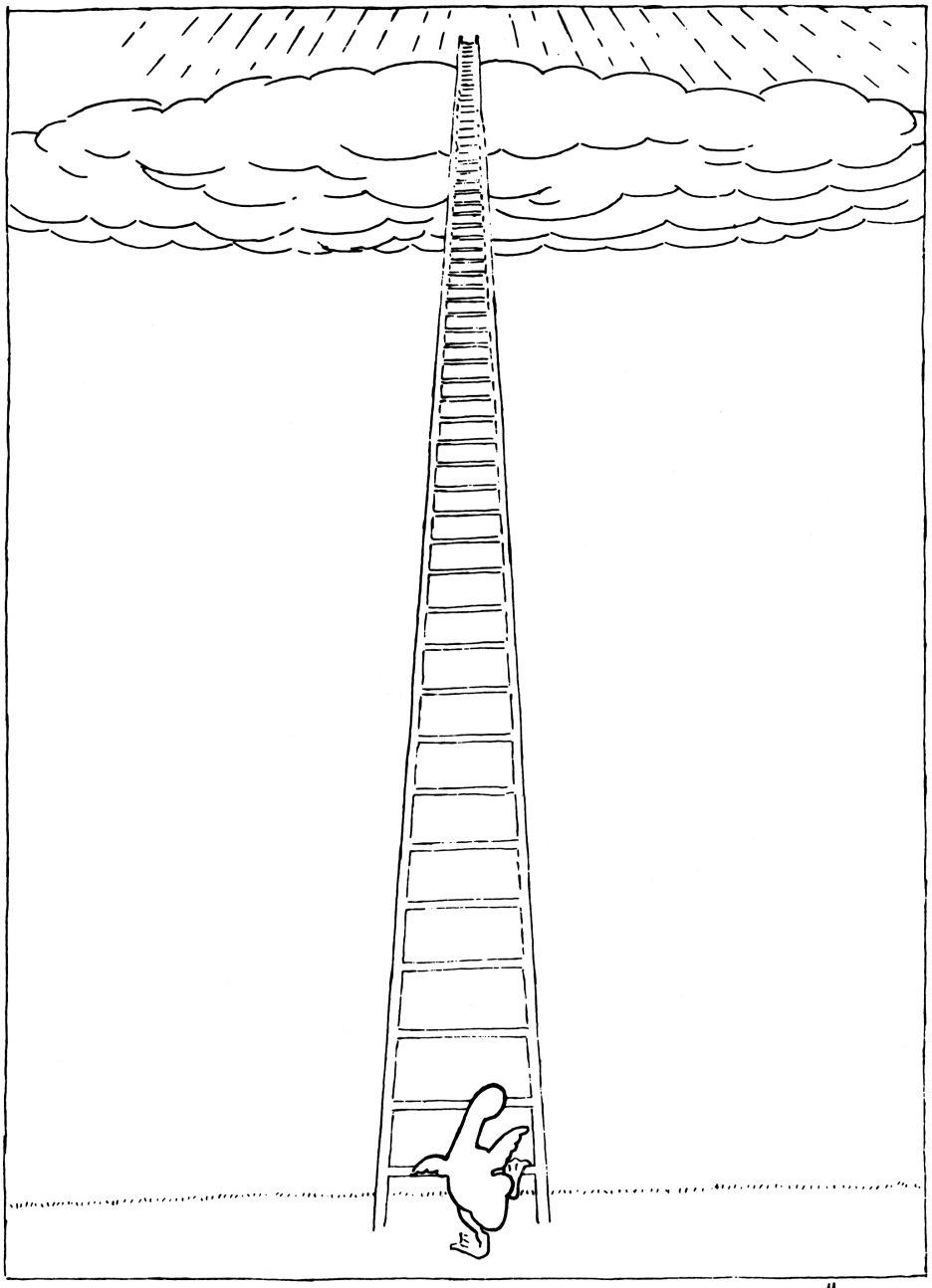

„NUR AUF STUFEN STEIGT MAN ZUR HÖHE."

OLAF GULBRANSSON
1873-1958

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GENERAL INTRODUCTION

Reproduction is the result of male and female interaction. Although both sexes have to cooperate to pass on their genes to next generations and to increase their share of a species' gene pool, they differ in strategies to optimize their reproductive output. Traditionally conflict between the sexes has been viewed to arise because males produce many small gametes (sperm) whereas females produce few large gametes (eggs) (Andersson 1994). Theoretically males can repeatedly mate with many females while females are limited in their opportunities to reproduce and their fitness can be significantly lowered by inappropriate matings (Trivers 1972). Because commonly females are the sex, which bears the greater cost of reproduction and reproductive error, they represent the limiting factor for reproduction. Therefore male-male competition over access to females (intrasexual selection) and female choice (intersexual selection) originates (Andersson 1994).

Sexual selection defined by Darwin (1871) as "selection that arises from differences in mating success" at first has been seen to occur exclusively "before parents unite" (Darwin 1871) and subsequent studies maintained this precopulatory emphasis (Andersson 1994). In a pioneering paper Parker (1970) called attention to the fact that male contest over reproduction may continue after insemination. In promiscuous species males compete via their ejaculates for fertilization of a given set of ova, a phenomenon described as 'sperm competition'. Postcopulatory male-male competition has subsequently been observed to ubiquitously occur from plants to mammals (Birkhead and Møller 1998).

It took nearly 25 more years until also the idea of cryptic female choice extended the possibility of female choice past initiation of copulation (Eberhard 1996). Female controlled processes and structures enable females to selectively influence paternity of males with particular traits even after insemination. In particular the potential female ability to select sperm within her reproductive tract in order to increase her fitness became a focus of scientific discussion (Birkhead 1998; Birkhead 2000; Eberhard 2000; Kempenaers et al. 2000; Pitnick and Brown 2000). However, so far the evidence for its

existence is ambiguous (e.g. insects: Wilson et al. 1997, Clark et al. 1999, Stockley 1999, Mack et al. 2002, Nilsson et al. 2003, Bretman et al. 2004; fish: Evans et al. 2003, Pilastro et al. 2004; lizards: Olsson et al. 1996; birds: Birkhead et al. 2004; but see Stockley 1997, Birkhead et al. 1999, Cunningham and Cheng 1999).

An extreme case of sexual conflict in the narrow sense (enhancement of reproductive success of one sex at the expense of fitness of the other sex (Parker 1979)) may occur in species where males are able to force females to copulate. This behavior is found in a variety of species (primates: Smuts and Smuts 1993; birds: McKinney et al. 1983; reptiles: Olsson 1995; fish: Farr 1980; insects: Thornhill 1980). Despite extreme behavioral resistance, females often appear to have no control over their copulatory partners. Cryptic female choice may than be a mechanism, which evolved to circumvent precopulatory male manipulation and to assure female reproductive optimum.

This thesis investigates male and female influences on paternity and focuses on evaluation of cryptic female sperm selection as a mechanism shaping male reproductive success. For this purpose we chose wild mallards, *Anas platyrhynchos*. In this duck species forced copulations represent a major component of the mating system (McKinney et al. 1978; McKinney et al. 1983), although mallards form socially monogamous pair bonds in autumn (Cramp 1977). Females show strong preferences for their social partner (Cunningham 2003) and offspring viability as well as mother productivity is significantly reduced when females are paired with non-preferred males (Bluhm and Gowaty 2004). However the possession of an intromittent organ facilitates forced copulations by extra-pair males (McKinney and Evarts 1997), which are always heavily resisted by the female (McKinney and Evarts 1997; Cunningham 2003). Nonetheless these extra-pair copulations can result in fertilization (Burns et al. 1980; Evarts and Williams 1987).

The main aims of this thesis were to investigate

1. *Evidence for cryptic female choice in wild mallard populations*
   We examine levels of extra-pair paternity in wild mallard populations and compared it to reported frequencies of extra-pair copulations. (Chapter One)
2. *Relative importance of cryptic female sperm selection and sperm competition in determining male fertilization success*

By inseminating groups of related females with a sperm mixture containing equal number of sperm from one brother and from one unrelated male in combination with measurement of sperm quality, we compare postcopulatory male and female influences on paternity. (Chapter Two)

3. *Potential mechanism of female influence on sperm competition*
   Sperm swimming speed is an important determinant in sperm competition. We look at differential female influence on this male trait. (Chapter Three)

Further aspects of male and female reproductive decisions were investigated during this study.

Male side to reproduction:

1. *Extra-pair paternity*
   Forced extra-pair copulations are well-documented in waterfowl. We report on the extra-pair fertilization success of this male reproductive strategy in wild mallard populations. (Chapter One)
2. *Trading copulation frequency against ejaculate quality*
   Production of ejaculates is a timely and costly process. We examine the costs of frequent copulations in terms of reduced competitiveness of single ejaculates. (Chapter Four)
3. *Testosterone and testis size*
   Testis size is correlated with sperm production in many species. We investigate if it is also related to production of the sex steroid testosterone, which is an important factor governing female mate choice and male reproductive behavior in mallards. (Chapter Five)

Female side to reproduction:

1. *Manipulation of primary sex ratios*
   If each sex provides different fitness benefits, gender of offspring will be an important determinant of female reproductive success. We determine primary sex ratios in wild mallards in general and in consecutive nesting attempts. (Chapter One)

2. *Brood parasitism*

   By laying eggs in foreign nests ducks can significantly increase their reproductive output. We quantify levels of brood parasitism in two wild mallard populations and relate it to nesting density as a potential constraining factor of egg dumping. (Chapter One)

Finally the appendix provides technical information on the microsatellites used for paternity and maternity assessment in this study.

CHAPTER ONE

GENETIC ANALYSIS OF SEX RATIOS, BROOD PARASITISM AND EXTRA-PAIR PATERNITY IN MALLARDS (*ANAS PLATYRHYNCHOS* L.)

Angelika G. Denk & Bart Kempenaers

ABSTRACT

Mallards, *Anas platyrhynchos*, are among the most common waterfowl species in the Northern Hemisphere. Despite their abundance and despite growing interest of behavioral ecologists and evolutionary biologists in key aspects of their behavior, few studies have used genetic tools to investigate their mating system. We studied the breeding biology of mallards by examining 41 clutches from two areas that differ in breeding density. We focus on three aspects of mallard reproductive behavior. First, adult sex ratios in mallards are often reported to be male-biased. In our population, the proportion of males observed during autumn and winter counts varied between 59% and 67%. Here we show that this bias is already present in the primary sex ratio: on average 60% of eggs in a clutch are males. Second, intra-specific brood parasitism is observed in many duck species. We found egg dumping in 53% of mallard clutches in a high breeding density area, whereas brood parasitism was entirely absent in an area with low breeding density. Third, although mallards are socially monogamous, forced extra-pair copulations are frequently observed. Using microsatellites, we estimate that a minimum of 56% of broods contained at least one extra-pair young. Overall, at least 14% of fertilized eggs were sired by an extra-pair male. Breeding density did not influence the proportion of broods with extra-pair paternity. However, broods from the high density area contained significantly more extra-pair young than broods from the low density area.

submitted to *AUK*, 17 November 2004

INTRODUCTION

Sexual selection is an important evolutionary agent shaping morphology, mating behavior and life history (Andersson 1994). Individuals face strong selection to pursue reproductive strategies that ultimately increase their fitness. Waterfowl, in particular ducks (*Anatinae*), exhibit three notable features of their reproductive system, which should be closely linked to reproductive success: male-biased sex ratios at the population level, egg dumping by female ducks and forced extra-pair copulations. Here, we report on a study of free-living mallards (*Anas platyrhynchos*), one of the most common waterfowl species in the Northern Hemisphere (Cramp 1977). We use molecular tools to investigate primary sex ratios as well as the occurrence of intra-specific brood parasitism and extra-pair paternity. The aim of our study is to provide a detailed description of the mating system of mallards and to discuss the adaptive value of individual reproductive decisions.

Sex ratios

Sex ratio manipulation is a classic example of an individual's reproductive decision that can be explained by evolutionary theory (Sheldon 1998). If daughters and sons provide different fitness benefits to their parents, selection should favor parents which produce the more profitable sex (Charnov 1982). A paradoxical situation arises in waterfowl populations, which are frequently observed to be male-biased (Bellrose et al. 1961; Gowaty 1993; Blums and Mednis 1996 and citations therein). Adult sex ratios of two to three or more males per female are recorded for canvasbacks (*Aythya valisineria*) and common pochard (*Aythya ferina*) (Rohwer and Anderson 1988; Oring and Sayler 1992). Also in mallards adult sex ratios with significant skews in favor of males are frequently reported, and it is unlikely that this can be attributed to pure sampling errors (see Fig. 1).

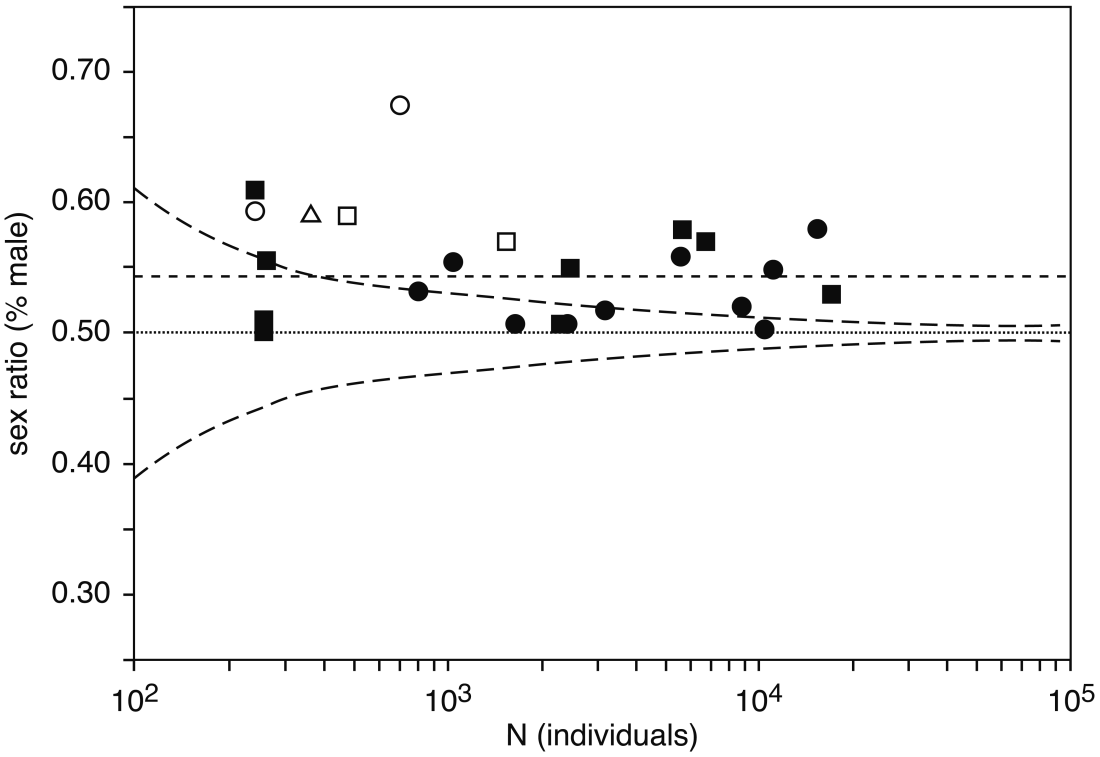

Figure 1. Population sex ratios of wild mallards. Data (filled symbols) are obtained from field observations reported in (Bellrose et al. 1961; Humburg et al. 1978 and Ferguson et al. 1981); filled circles: all data; filled squares: data restricted to counts in March and April; counts obtained from trapping and hunting are excluded due to potential bias). Open symbols show adult sex ratios observed in this study at Lake Starnberg and Lake Ammer (circles: October count; squares: January count). The triangle indicates the primary sex ratio (this study). The dotted line indicates the null hypothesis of parity; curved, long-dashed lines display binomial significance levels (p=0.05, two-tailed; as described in Palmer (2000)). The medium-dashed line represents the mean of the observed adult sex ratios.

This bias is particularly puzzling because many waterfowl species, including mallards, form socially monogamous pairs (Cramp 1977). Although alternative reproductive behaviors such as forced extra-pair copulations exist, observations suggest that this behavior is not predominantly pursued by unpaired males (Goodburn 1984; Davis 2002a;

Cunningham 2003). Therefore it can be assumed that unpaired males suffer reduced reproductive success and an overproduction of males seems to be maladaptive.

A first step in understanding the paradox of unbalanced population sex ratios in species with a monogamous mating system, is to locate the origin of the bias. The observed bias in adult sex ratios can be caused by skewed brood (primary) sex ratios, sex-dependent hatching success or sex-differential mortality after hatching (Bellrose et al. 1961). Differential parental investment during rearing presumably plays only a tangential role in species with precocial young. So far, no information about the primary brood sex ratio from free-living duck populations is available. Only a few studies examined sex ratios in various duck species at hatching, but they failed to detect a deviation from 50:50. However, in most of these studies clutches were artificially incubated, hatched in an incubator (Sowls 1955; Mendall 1958; Bellrose et al. 1961; Swennen et al. 1979) or were produced by ducks kept in captivity (Dubovsky 1990). Only (Blums and Mednis 1996) determined the sex of newly hatched Northern shovelers (*Anas clypeata*), common pochards and tufted ducks (*Aythya fuligula*) in wild populations, but found no evidence of parental manipulation of brood sex ratios. In contrast, sex differential mortality after hatching reportedly affects more males (for mallards: Bellrose et al. 1961). This, in combination with a higher hunting pressure on males, should cause a female biased adult sex ratio (Bellrose et al. 1961). Thus, the origin of male-biased sex ratios in adult ducks remain obscure (Blums and Mednis 1996). Information on primary sex ratios is clearly needed to further investigate the adaptive value of male-biased sex ratios in waterfowl. In the present study we investigate whether the male-biased sex ratio observed in adult populations is already caused by a bias in the primary brood sex ratio.

Brood parasitism

Intraspecific brood parasitism or egg dumping is a common phenomenon in waterfowl (Yom-Tov 1980; Rohwer and Freeman 1989; Sayler 1992; Yom-Tov 2001). Females can substantially increase their reproductive output by parasitizing conspecific nests (Ahlund and Andersson 2001), often at negligible fitness costs to the host (Dugger and Blums 2001; Blums and Clark 2004). Hosts may even benefits from egg dumping via increased offspring survival (Eadie and Lumsden 1985), or through increased inclusive fitness if

host and parasite are related (Andersson 2001). Clear fitness benefits for parasites in combination with low costs for hosts raise the question why this behavior is not more common and why females differ in their reproductive tactic. There is evidence that tactics are flexible and might change between years (Ahlund and Andersson 2001 and citations therein). Additionally, females might be constrained by the availability and detectability of nests to parasitize. Therefore the frequency of brood parasitism should be positively related to nesting density (Yom-Tov 2001).

Despite abundant studies on brood parasitism in waterfowl (Rohwer and Freeman 1989; Sayler 1992; Yom-Tov 2001) data on the frequency of egg dumping based on genetic markers are still scarce. Most studies are based on indirect assessment of brood parasitism, e.g. comparing egg size, shape and color, checking number of eggs in a nest and egg laying frequency, or on direct behavioral observations (e.g. Dugger and Blums 2001; for mallards: Bengtson 1972; Titman and Lowther 1975). These methods might underestimate the frequency of brood parasitism. Molecular methods are essential to precisely assess levels of brood parasitism and its adaptive significance. Here, we compare the frequency of brood parasitism in two mallard populations that differ in breeding density.

Extra-pair paternity

The use of molecular tools revealed that extra-pair paternity occurs in nearly 90% of approximately 130 examined bird species (Griffith et al. 2002), but only few estimates of extra-pair paternity exist for waterfowl (Anserinae: Choudhury et al. 1993; Dunn et al. 1999; Kraaijeveld et al. 2004; Larsson et al. 1995; Anatinae: Evarts and Williams 1987; Triggs et al. 1991; Peters et al. 2003). The percentage of waterfowl broods containing extra-pair young ranges from 0% in barnacle goose (*Branta leucopsis*; Choudhury et al. 1993; Larsson et al. 1995) and blue duck (*Hymenolaimus malocorhynchos*; Triggs et al. 1991) to 40% in black swan (*Cygnus atratus*; Kraaijeveld et al. 2004) and at least 48% in mallards (Evarts and Williams 1987).

Forced extra-pair copulations are well-documented in waterfowl (reviewed by McKinney and Evarts 1998; McKinney et al. 1983). However, it remains largely unknown whether forced extra-pair copulations result in fertilizations and whether this

male behavior is a successful alternative reproductive strategy. Dunn et al. (1999) found that despite high percentages of extra-pair copulations (46-56 % of all attempted copulations) extra-pair paternity was low in Ross' (*Chen rossi*) and lesser snow geese (*Chen caerulescens*) (only 2-5% of young). Potential female strategies (e.g. sperm ejection, cryptic sperm selection) or defenses by the social mate (e.g. forced within pair copulations) may counteract forced extra-pair-copulations. To assess the adaptive relevance of these strategies more data on extra-pair fertilization success in wild populations are necessary. Here we estimate the frequency of extra-pair paternity in mallard populations, relate it to breeding density and compare it to other studies on this species.

MATERIAL AND METHODS

Study site and nest detection

From April-June 2001 we searched for mallard nests at Lake Starnberg (47º54'N, 11º18'E) and Lake Ammer (48º00'N, 11º08'E), Southern Germany. At Lake Starnberg we focused on the Island of Roses (24 ha), a favorite waterfowl breeding ground with high nesting densities (mean inter-nest distance 69 m). At lake Ammer we searched along the shore, where the density of mallard nests was low (mean inter-nest distance 1167m).

To increase the chances of finding nests and to facilitate capture of the female we distributed 40 wooden nest-boxes (dimensions: 50 cm x 30 cm x 30 cm) along the shores. The front side of the box could move up and down along two metal rails. The front was opened about two-thirds and prevented from falling down using a wooden pin. We attached a rope (6 m) to the pin, which enabled us to close the nest-box from a distance and capture the female on the nest. We established the nest-boxes on wooden artificial islands (1m x 1m) and on protected spots on land close to the waterline (e.g. on piers of boathouses). Six nest-boxes were accepted by mallards and used for breeding. Other nests were found by observing female and male behavior, by walking along potential breeding grounds and by hints from private people (e.g. two duck nests were found in the loft of an inhabited house).

Each discovered nest was visited every second day until the female started incubation and all eggs were warm. After the (assumed) onset of incubation or when an incubated nest was discovered, all eggs were collected under license and egg length and width were measured with a caliper; egg volume was calculated following Hoyt (1979). Eggs were checked for signs of a developing embryo by candling with an ORBAN candling lamp (Tempo Nr. 119). If a developing embryonic disc was visible, the egg was put in a –20ºC freezer for an hour, and then opened. If development was not recognizable beyond doubt, eggs were incubated for three more days in a computer-assisted motor-incubator (SV250, J. Hemel Brutgeräte, Verl-Kaunitz, Germany) before opening. All embryos were stored in 70% ethanol until DNA extraction. Age of the embryos was estimated (Freeman and Vince 1974) and the onset of laying was estimated under the assumption that one egg was laid per day.

Incubating mallards cushion the nest bowl with their own down feathers (Cramp 1977). To establish maternity, we collected down feathers from each nest and stored them in 70% ethanol until DNA extraction.

From each female caught on the nest (N=9), we took a blood sample (100 µl) and we measured bill length (from tip of the bill to the start of the feathers) and tarsus length using calipers and foot size using a ruler. All measurements were taken to the nearest millimeter. Blood samples were diluted in Queen's lysis buffer [0.01 M Tris, 0.01 M NaCl, 0.01 M Na-EDTA, 1% *n*-Lauroylsarcosine; adjusted to pH 8.0]. We marked females individually with a combination of two colored plastic bands (A.C. Hughes Ltd, Hampton Hill, UK), and attached a radio-transmitter (TW-4 single button celled tag, Biotrack Ltd, Wareham, UK) to the base of the tail feathers using two cable ties and a drop of superglue (Loctite gel, Henkel, Germany) (Giroux et al. 1990). Two ducks lost the transmitter before they initiated a second clutch, but they could be identified by their plastic bands. One female disappeared from the study area. The other six females were relocated using radio-tracking (receiver: TRX1000S; three-element-yagi-antenna; Wildlife Materials Inc., USA).

Four out of eight females were found on a second nest and two females produced a third clutch (all collected). Two nests from which only feathers were collected belonged to the same female (based on the microsatellite analysis, see below). In spring 2002,

another first and replacement clutch from a new individually marked female were collected and included in this study. Thus, a total of eight replacement clutches from six different females were analyzed.

DNA extraction and preparation

We used DNeasy® Tissue Kit (Quiagen) to extract genomic DNA from embryos and from the basal part of the feather's calamus, following the manufacturer's protocol. We extracted genomic DNA from blood samples using GFX^{TM} Genomic Blood DNA Purification Kit (Amersham Pharmacia Biotech Inc., USA) also according to the manufacturer's protocol. We followed a standard PCR protocol (10µl reaction) using 1µl genomic DNA, 100µm dNTP, 0.12µl of a 1% Bovine Serum Albumen, 1.5 mM MgCl2, 10 mM 10x Mg-free buffer (Promega) and 0.25 U of Taq DNA polymerase (Promega).

Sex determination and parentage analysis

To determine the sex of the embryo we used the P2/P8 sexing primers described by (Griffiths et al. 1998). In the PCR profile, an annealing temperature of 48°C was used in 25 amplification cycles. DNA of a female duck was successfully used in each PCR as a positive control. To further test for the reliability of the sexing primers in mallards we sexed 19 adult ducks of known sex (ten males, nine females). Molecular assignment was identical to the morphological evidence in all cases.

For assessment of maternity and paternity we used seven polymorphic microsatellite markers developed for wild mallards (Denk et al. 2004). The annealing temperature was set at 60°C and the number of amplification cycles varied between 30 (embryonic DNA / DNA from blood) and 35 (feather DNA). Loci, which showed a mismatch to the maternal genotype (see below), were rerun to prevent erroneous maternal exclusion. Mismatching alleles were only accepted after two runs yielded identical results. All amplified fragments were resolved on an ABI Prism 310 Genetic Analyzer (Applied Biosystems).

Genotypes obtained from the blood of incubating females caught on the nest were all identical to the genotypes of feathers collected from the same nest (N=9). Thus, we

further assumed that feathers collected in the nest bowl stem from the incubating female and can be used for maternity assessment.

To evaluate maternity we compared the genotypes of all eggs from a nest with those of the putative mother. Embryos that showed mismatches at more than one locus (range: 2-7) were considered the result of brood parasitism. In 2.3% of eggs (N=8), there was only one mismatch with the putative mother's genotype and these were considered the result of mutation. The exclusion probability of the set of seven microsatellite loci was high ($p>0.999$).

Because we do not have information on the social mates of the females included in this study, we can only obtain minimum estimates of the frequency of extra-pair paternity, as follows. We first excluded all eggs that resulted from brood parasitism. Then, we considered a nest to contain extra-pair young if we found at least three paternal alleles at two or more loci among the remaining eggs. We further assumed that the two most common paternal alleles stemmed from the social partner. If an egg differed at two or more loci from the most common paternal alleles, we considered it an extra-pair young. In five nests the variability of alleles was too high to construct a hypothetical father. These clutches were included to determine the frequency of broods with extra-pair paternity, but were excluded from all analyses on the frequency of extra-pair paternity within broods.

In total we collected 44 mallard clutches. Three nests were excluded from further analyses, because the genotypes of all eggs differed from those of the feather collected at that nest. Although it is not impossible that 100% of eggs were dumped (see results), we assumed that in these cases the feather did not belong to the incubating female. The remaining 41 clutches contained a total of 365 eggs, of which 350 (96 %) contained an embryo. There was no seasonal pattern in the occurrence of clutches with eggs that failed to develop (logistic regression: $\chi^2=0.35$, df=1, $p=0.55$).

Adult sex ratios

To estimate the adult sex ratio in the mallard populations at Lake Starnberg and Lake Ammer, we counted adult female and male mallards once in autumn 2001 (on the 12th, resp. the 10th of October) and once in the following winter 2002 (on the 2nd , resp. the 3rd

of January). Ducks were counted from 28 observation spots at Lake Starnberg and from 21 spots at Lake Ammer using a KOWA TSN-1 telescope (20x magnification, wide angle lens). We counted ducks on the water as well as those resting on land.

To assess the impact of hunting on sex-differential mortality, we sent out questionnaires to all registered hunters at both lakes, but only obtained data from the southern half of Lake Starnberg.

Statistical Analyses

We tested the deviation of the primary sex ratio from parity using a one-sample t-test. The unit of analysis is the clutch, which is defined as all the eggs sired by the incubating female. Only the first clutch found per female was used and all clutches with more than two eggs were included in the analysis. Binomial tests were used to test the deviation of adult sex ratios from 50:50.

In analyses with sex ratios of individual broods as dependent variable, we used generalized linear models (GLM) to account for the binomial structure of the data. We specified binomial errors and a logit link function, with number of males per clutch as dependent variable and clutch size as the binomial denominator. A generalized linear mixed model (GLMM) was used to analyze changes in the sex ratio between first and replacement clutches (female identity as random factor). To test effects of maternal body size on sex ratio, we used the first principal component (PC1), which explains 71.3% of variation in bill, tarsus and foot length, as response variable in a GLM. We looked at sex-specific differences in egg size using mixed models (REML) controlling for female respectively date effects.

For all statistical analyses we used SPSS (12.0.1) or Genstat 7.1.0.198 (Genstat 2003). All tests are two-tailed.

RESULTS

General breeding ecology

The total number of eggs in the nest was lower in the high density nesting area, although not significantly so (Table 1). However, when excluding eggs that resulted from brood

parasitism, clutch size was significantly lower in the high density area (Table 1). Because nests in the low density area were found earlier in the season, we restricted the dataset to nests collected within the same time window (N=26) and obtained the same result (Table 1). The same trend was found when statistically controlling for the seasonal decline in egg numbers over the entire reproductive season (GLM; eggs per nest: laydate: $F_{1,31}$=-2.96, p=0.006, breeding density: $F_{1,31}$=-0.12, p=0.91; clutch size: laydate: $F_{1,31}$=-1.92, p=0.064, breeding density: $F_{1,31}$=1.85, p=0.074). Egg volume also differed between the two areas, but did not decrease seasonally (Table 1; GLM: laydate: $F_{1,31}$=-1.07, p=0.29, breeding density: $F_{1,31}$=2.13, p=0.041).

Replacement clutches (N=6) were similar sized (paired t_5=-0.567, p=0.60), but contained larger eggs (paired t_5=-7.138, p=0.001) than first clutches. This pattern was also found when the two second replacement clutches were included (REML; clutch size: Wald F_1=0.01, p=0.91; egg volume: Wald F_1=3.91, p=0.048).

All egg measurements were highly repeatable within females (length: r=0.99, $F_{33,194}$=5209.9, p<0.001; width: r=0.99, $F_{33,194}$=5167.5, p<0.001; volume: r=0.84, $F_{33,194}$=36.8, p<0.001; n_0=6.4). Egg volume was independent of the sex of the egg (REML: Wald F_1=0.59, p=0.44) and did not differ between eggs belonging to the incubating female and those that were dumped (paired t-test: t_{17}=-1.491, p=0.15). Furthermore, assumed within-pair eggs did not differ from extra-pair eggs in volume (paired t-test: t_{16}=-1.315, p=0.21).

	High density	Low density	Statistical test
Laydate	134±25	100±19	t_{39}=3.25, p=0.002
Number of eggs in nest[a]	7.8 ± 3.9	9.7 ± 2.1	t_{32}=-0.76, p=0.46
Clutch size[a, b]	5.8 ± 3.2	9.7 ± 2.1	t_{32}=-2.26, p=0.031
Number of eggs in nest[c]	8.8 ± 4.2	9.7 ± 2.1	t_{24}=-0.52, p=0.61
Clutch size[b,c]	6.5 ± 3.5	9.7 ± 2.1	t_{24}=-2.30, p=0.032
Mean egg volume (cm^3)[a]	46.7 ± 4.1	53.3 ± 6.4	t_{32}=-3.17, p=0.003
Proportion of parasitized nests	0.53 (34)	0 (7)	Fisher's exact p=0.026
Proportion of broods with EPY	0.59 (34)	0.43 (7)	Fisher's exact p=0.68
Mean proportion of EPY per clutch	0.13 (31)	0.06 (5)	GLMM Wald F_1=6.77, p=0.009

[a] All first breeding attempts included.
[b] Number of eggs belonging to the incubating female
[c] Dataset restricted to 15. March 2001-14. May 2001

Table 1. Comparison of reproductive parameters of mallards breeding at high (Island of Roses) and low density (Lake Ammer).

Sex ratios

We determined the primary sex ratio of 30 clutches (all first nests containing more than two eggs laid by the incubating female). On average, 60% of eggs were males (range: 0% (0/5)-100% (3/3)), which was significantly different from parity (one-sample t-test: t_{29}=3.28, p=0.003). In six clutches there were eggs (1-4) that did not show any sign of embryonic development. Excluding these nests does not change the conclusions (62% males in N=24 nests, t_{23}=3.01, p=0.006). No seasonal effect on sex ratios was detected (GLM: $F_{1,29}$=0.24, p=0.63), and the sex ratio of first and replacement clutches did not differ (GLMM: Wald F_1=2.01, p=0.16). Sex ratio was independent of clutch size (REML: Wald F_1=1.13, p=0.29) and mean egg volume (REML: Wald F_1=0.18, p=0.68), but larger females produced significantly more sons in their first clutch (Fig. 2).

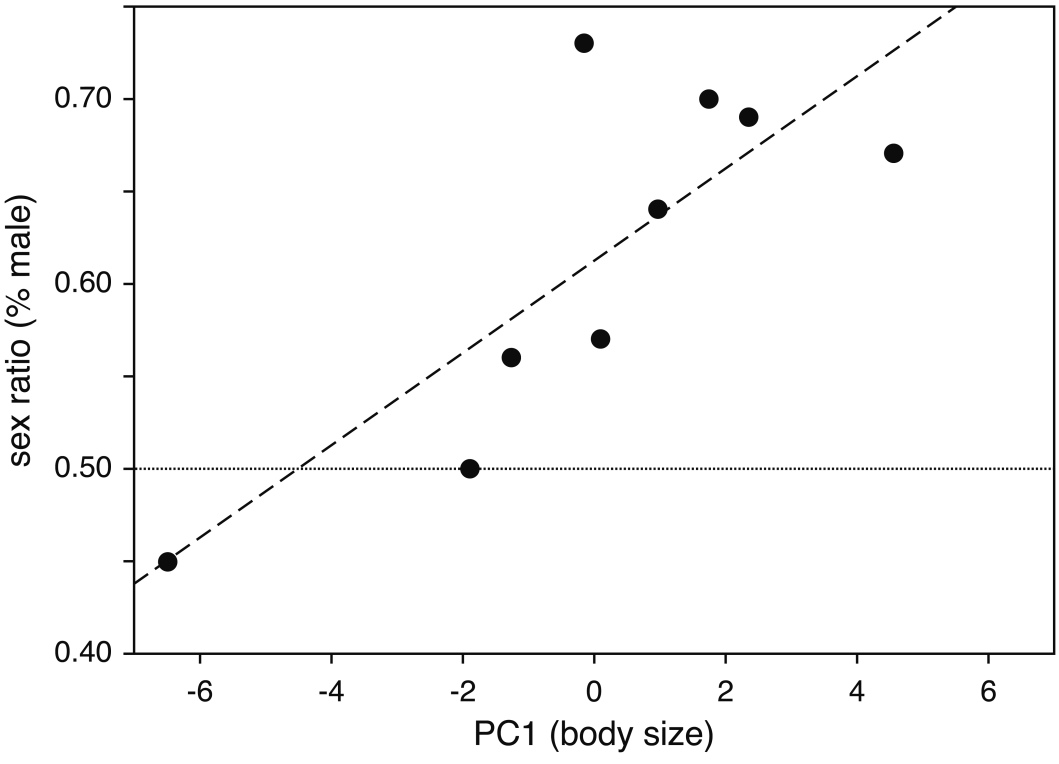

Figure 2. The relationship between female size (first principle component of bill, tarsus and foot length) and the brood sex ratio (GLM: $F_{1,8}$=10.05, p=0.019).

The observed primary sex ratio corresponded with the observed adult sex ratios (Fig. 1; Table 2). Adult mortality caused by human impact was greater for male mallards. In the period between 27 October 2001 and 15 January 2002, hunters shot 42 mallards at the southern end of Lake Starnberg, of which 35 (83%) were males (binomial test: $p<0.01$).

	Lake Starnberg	Lake Ammer
October 2001	59% (241)	67% (701)
January 2002	59% (473)	57% (1540)

Table 2. Sex ratios (% males) of adult mallard populations at Lake Starnberg and Lake Ammer in autumn 2001/winter 2002. Total counts are indicated in brackets (number of individuals).

Intraspecific brood parasitism

The frequency of egg dumping was markedly different in the two areas (Table 1); no brood parasitic eggs were found in the low breeding density area. In the high-density area on average 19% of eggs in a nest were dumped (range: 0-94%; Fig. 3). The percentage of dumped eggs in this area did not change with laying date (Spearman rank: $r=-0.041$, $N=34$, $p=0.82$). The average sex ratio of dumped eggs per nest was 43% (range: 0-100% males), but did not differ from parity (one-sample t-test: $t_{17}=-0.808$, $p=0.43$) and was not significantly different from the sex ratios produced by the incubating females (GLM: $F_{1,51}=-1.32$, $p=0.19$).

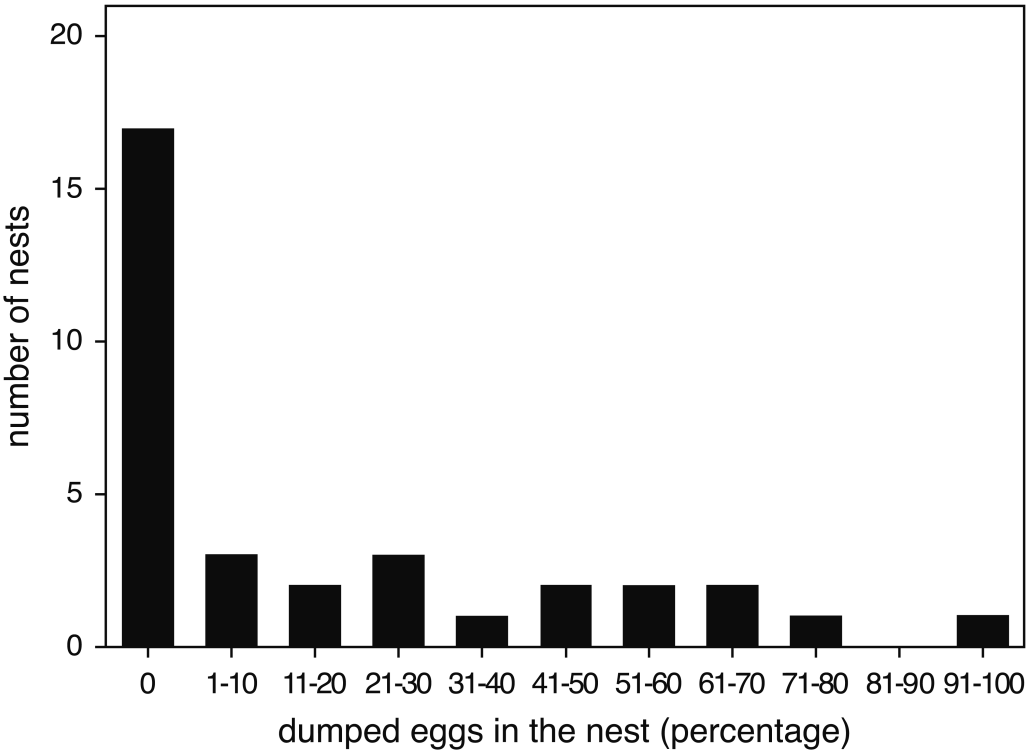

Figure 3. Frequency distribution of the levels of intraspecific brood parasitism in mallard nests from a high density area (Island of Roses, N=34 nests). No brood parasitism was observed in an area with low breeding density.

The number of eggs produced by the incubating female did not differ between nests with and without parasitic eggs (with parasitic eggs: 5.5±3.5; without parasitic eggs: 6.3±3.0; t_{32}=0.399, p=0.69), suggesting (a) that parasitic females did not remove eggs before laying their own, and (b) that females did not lay fewer eggs in response to parasitism. However, two females were observed incubating clutches to which they contributed little (1 out of 18 and 2 out of 10 eggs). Overall, only two parasitic ducks could be identified; both females first dumped eggs in foreign nests before starting their own clutch.

In two mallard nests in the high density area (Island of Roses) we detected interspecific brood parasitism. In each nest we found two eggs of red-crested pochard (*Netta rufina*), a species which was observed to breed on the island in close proximity to mallards (minimum distance observed: 5m).

Extra-pair paternity

Of 41 nests examined in this study, 56% (23 nests) contained at least one extra-pair young (EPY). Overall, 14% of 230 young were sired by extra-pair males (range: 9% (1/11) - 50% (7/14; Fig. 4). The proportion of nests containing extra-pair young did not differ between the high and low breeding density area (Table 1). However, the average proportion of extra-pair paternity within broods was significantly higher in clutches from the high breeding density area (Table 1). The proportion of extra-pair young in a clutch did not change over the season (Pearson correlation: r=-0.090, N=41, p=0.60).

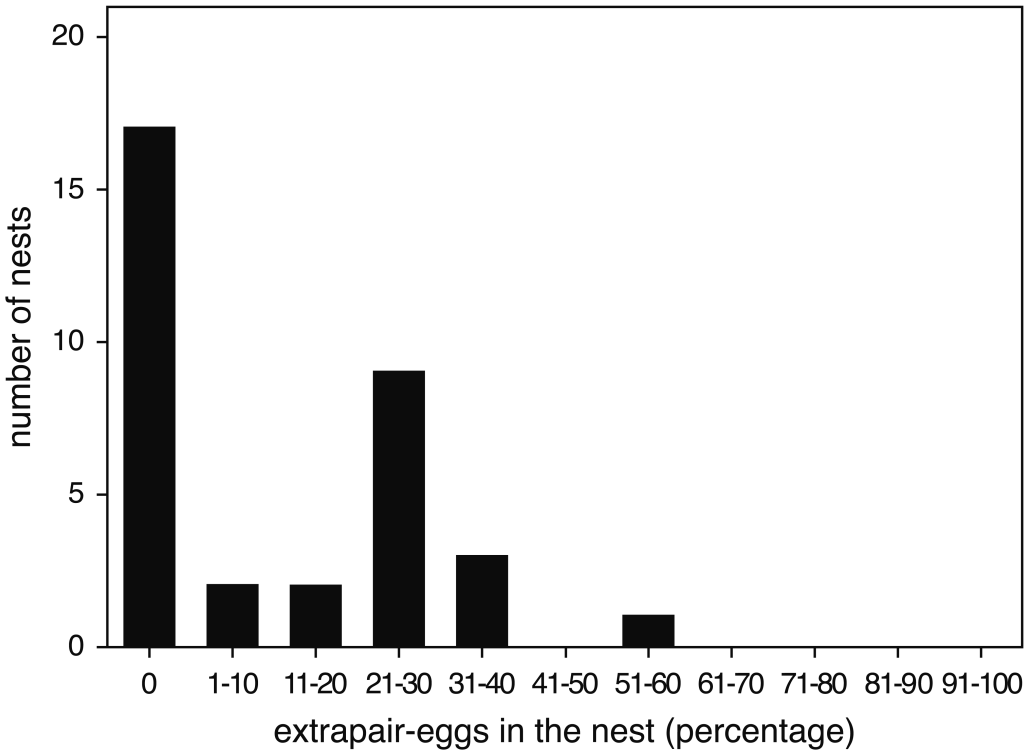

Figure 4. Frequency distribution of the proportion of extra-pair young in mallard clutches (high and low density areas combined; N=36 clutches).

Broods with or without extra-pair young did not differ in egg volume (t_{32}=1.136, p=0.26), nor in sex ratio (GLM: $F_{1,33}$=0.72, p=0.40). Furthermore, the sex ratio of extra-pair

offspring did not differ from that of their within-pair nest mates (GLMM: Wald F_1=0.21, p=0.65).

DISCUSSION

This study provides basic information on the mating system of mallards based on microsatellite analysis of 41 nests. The focus of this study was threefold. First, we examined the primary sex ratio, and showed that it is already male-biased, corresponding to the adult sex ratio. Second, we investigated the frequency of brood parasitism, comparing two areas with different breeding density. In the high density area, more than half of the nests contained dumped eggs, whereas parasitism was absent in the low density area. Third, we estimated the level of extra-pair paternity: at least 56% of nests contained extra-pair young. Although our data underestimate the true level of extra-pair paternity, this is the highest level reported in waterfowl so far.

Sex ratio

In birds the significance of adaptive sex ratio manipulation is still debated (for review see Hasselquist and Kempenaers 2002; Komdeur and Pen 2002) and so far the mechanisms of sex ratio manipulation are unknown (Hasselquist and Kempenaers 2002). At the individual level, evidence is accumulating that birds adaptively manipulate their offspring sex ratio according to various factors (e.g. female condition (Nager et al. 1999; Velando 2002), pairing status (Westerdahl et al. 2000; Ewen et al. 2001; Green 2002), paternal attractiveness (Burley 1981; Ellegren et al. 1996; Svensson and Nilsson 1996; Kolliker et al. 1999), environmental conditions (Appleby et al. 1997; Torres and Drummond 1999), local recruitment (Komdeur et al. 1997; Ewen et al. 2003), hatching order (Arnold et al. 2001; Badyaev et al. 2002) and seasonal effects (Smallwood and Smallwood 1998); but see (Bensch et al. 1999; Saino et al. 1999; Ramsay et al. 2003; Zann and Runciman 2003)).

In contrast, evidence for sex ratio bias at the population level is rare and reported deviations from parity seem to fall within binomial sampling variation (Palmer 2000). Waterfowl seem to form an exception in that male-biased sex ratios at the population

level are frequently reported (Bellrose et al. 1961; Gowaty 1993; Blums and Mednis 1996 and citations therein). For mallards, Figure 1 clearly shows that the observed population sex ratios outrange pure binomial sampling error. However, the data shown in Figure 1 can be misleading and should be interpreted cautiously. Male and female mallards differ in their migratory behavior (Perdeck and Clason 1983) and females disappear from population counts during incubation, which could explain the observed male-biased sex ratios. However, close inspection of the data suggests that the male bias is present, irrespective of the timing of population counts. For example, population counts during March-April, the time of pair arrival at the breeding grounds, also show male-biased sex ratios (Fig. 1). Moreover, there are no counts that show a female-biased sex ratio.

So far, the underlying mechanism leading to an excess of adult male mallards is unclear. Our study suggests that the primary sex ratio is already significantly male-biased and closely approximates the adult sex ratios. Our result contradicts a study on domestic strains for commercial mule duck production (Muscovy duck (*Cairina moschata*) x Peking duck (*Anas platyrhynchos*)), which did not find a sex ratio bias at early stages of incubation and reported that the unbalanced adult sex ratio is due to sex-differential mortality just prior to or at hatching (Batellier et al. 2004). However, mortality estimates were based on developing hybrid ducklings and higher mortality of female embryos/young ducklings may be a consequence of intergeneric crosses rather than reflecting a general, natural process.

Although our study suggests that in the wild female mallards produce more male than female eggs, the adaptive value of this behavior remains unclear. Mallards are socially monogamous and forced extra-pair copulations are pursued by mated (Goodburn 1984) as well as unmated males (Davis 2002a; Cunningham 2003), arguing against an alternative reproductive strategy specific for unmated males. Therefore, the production of a surplus of males does not seem to be adaptive. Although adult mortality might be higher for males (Bellrose et al. 1961; this study), adult sex ratios remain male biased. More studies on mallards and other waterfowl are necessary to test the generality of a male-biased primary sex ratio.

Although there was no consistent seasonal change in sex ratio, or a change from first to replacement clutches, female mallards significantly increased egg size in their

repeated breeding attempts – a pattern also observed in captive mallards (Batt and Prince 1979). Several studies show that ducklings from larger eggs are bigger at hatching (mallards: Batt and Prince 1979, Rhymer 1988; other waterfowl species: Dawson and Clark 1996; Erikstad et al. 1998; Badzinski et al. 2002; Pelayo and Clark 2003). They grow faster during the first critical days and weeks (Cox et al. 1998; Erikstad et al. 1998; Anderson and Alisauskas 2002) and have a higher survival probability (Dawson and Clark 1996; Cox et al. 1998). Improved locomotion, feeding efficiency, thermoregulation and reduced predation risk are assumed to positively influence survival of ducklings from large eggs (Rhymer 1988; Dawson and Clark 1996; Cox et al. 1998; Anderson and Alisauskas 2002). Cunningham and Russell (2000) found that female mallards even increase egg size according to their partner's attractiveness. Similarly, female mallards may increase egg size in later breeding attempts to increase survival chances of later born offspring.

Brood parasitism

Intra-specific brood parasitism is very common among Anseriformes: it is reported in 74 species, or 70% of species breeding in the western Palearctic and North America (Yom-Tov 2001). It can be assumed that egg dumping will be found in more Anseriformes species, as they will be investigated (Sayler 1992; Yom-Tov 2001).

Using microsatellite analysis we investigated brood parasitism in two mallard populations differing in breeding density. We detected a high level of egg dumping in an area with high nesting density (53%, Table 1), and no brood parasitism in an area where nests are spread out. The level of brood parasitism reported here is higher than that previously reported for other crowded populations of mallards, based on indirect maternity assessment (11-21%: Titman and Lowther 1975; Deubert et al. 1983). A study of an Iceland mallard population found only 1% of nests with dumped eggs (Bengtson 1972). Although breeding density was not reported, the given information suggests that it was low (approx. 30 nests/year at a lake of 38 km^2). Other observations of dispersed mallard nests suggested that levels of brood parasitism are low (1% of nests: Rohwer and Freeman 1989).

Our findings support the hypothesis that brood parasitism is more common when nests are crowded, like in colonies or on islands (Yom-Tov 1980; Rohwer and Freeman 1989; Yom-Tov 2001). This could be because (a) the detectability of potential host nests is higher and (b) nesting sites might be limited (Sayler 1992). Accordingly in wood ducks Semel et al. (1988) found that brood parasitism was strongly affected by visibility and density of nest boxes in artificial nesting structures.

Our data suggest that mallards did not simply dump eggs in nests of their neighbors. In only two out of 18 nests we were able to identify the parasitic female, which later nested on the same island. This indicates that parasitic females either did not nest at all, or nested away from the colony (island). This might be an adaptive strategy, because females under high breeding densities suffered a significant reduction in reproductive output compared to females nesting alone (compare clutch size in Table 1). Therefore, to optimize fitness, female mallards should nest in an area of low density and visit crowded nesting sites for parasitism. Further investigations are needed to compare reproductive success of individuals breeding in high/low density areas, and to find out which females are parasitic. As Ahlund and Andersson (2001) suggested tactics may alternate between years and different reproductive strategies might be determined by age and experience of a female.

Extra-pair paternity

Extra-pair paternity is widespread in birds (Griffith et al. 2002) and there is evidence in some species that females actively seek extra-pair copulations (e.g. Kempenaers et al. 1992; for review see Westneat and Stewart 2003). However, in waterfowl forced extra-pair copulations are frequently observed (McKinney et al. 1983; McKinney and Evarts 1998). In mallards these copulations are always heavily resisted by the female (Cunningham 2003) despite high costs (e.g. physical injuries; McKinney and Evarts 1998). Still little information is available on the fertilization success of these forced extra-pair copulations in mallards or in wild waterfowl populations in general (e.g. Dunn et al. 1999). Based on microsatellite analysis we found extra-pair young in at least 56% of nests, which is slightly more than estimated by Evarts and Williams (1987) using allozymes (48 %, N=31 nests). Although extra-pair paternity was observed both under

high and low breeding density, the proportion of extra-pair young was significantly higher in the crowded situation, a phenomenon also observed in various other bird species (for review see Westneat and Sherman 1997).

A minimum of 14% of all young were sired by an extra-pair male. This is less than expected based on the observed frequency of forced extra-pair copulations in a wild population, which showed a ratio of approx. 2 within-pair copulations (1.38/day) to 1 extra-pair copulation (0.78/day) (Cunningham 1997). Some male mallards seem to effectively protect females from sexual coercion (Goodburn 1984; Davis 2002b), so one could argue that only broods with at least one extra-pair young should be considered. In that case, the frequency increases to 24% extra-pair young, which is still below the expected level based on the overall copulation ratio. A similar pattern has been observed in Ross's and lesser snow geese, where frequent forced copulations resulted in low levels of extra-pair paternity (Dunn et al. 1999). To understand the reasons for the low success of forced copulations, further studies combining behavioral observations of mated pairs with molecular analyses would be useful. It also remains unknown what the costs and benefits are of those forced extra-pair copulations that lead to fertilizations, e.g. in terms of offspring fitness.

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CHAPTER TWO

PATERNITY IN MALLARDS: EFFECTS OF SPERM QUALITY AND FEMALE SPERM SELECTION

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ABSTRACT

Postcopulatory processes might play an important role in sexual selection. In theory, fertilization success could be controlled by females via selection of particular sperm within their reproductive tract, or it could be determined by sperm competition *per se*. In practice, these two mechanisms are difficult to disentangle. To assess the relative importance of both mechanisms we used artificial insemination in combination with measurements of sperm quality (swimming speed and motility) in mallards. In this species, females often lack behavioral control over copulations and hence may use postcopulatory mechanisms to optimize their reproductive output. One important factor affecting female fitness may be selection of genetically compatible males. To investigate the influence of sperm quality and parental relatedness on paternity we artificially inseminated twelve groups of related females with a sperm mixture containing equal numbers of sperm from a brother and from an unrelated male. Paternity was independent of the relatedness of the siring male to the female, but was significantly affected by long-term sperm swimming speed and motility. No interaction between relatedness and sperm quality on paternity was observed. These results suggest that female mallards are not able to select sperm on a purely genetic basis and emphasize the importance of sperm quality in gaining paternity.

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INTRODUCTION

Darwin first described sexual selection as an important evolutionary force acting through differential reproductive success of individuals (Darwin 1871). Traditionally, this process was perceived to occur exclusively precopulatory (Andersson 1994). However, as a consequence of female promiscuity – now known to be widespread in animals (Birkhead and Møller 1998) - sexual selection does not stop at insemination but continues after copulation (Birkhead and Pizzari 2002). Initially, research focused on male-male competition over fertilization (sperm competition; Parker 1970). However, in recent years the question of whether and to what extent females are able to bias paternity in favor of a particular male after having copulated with several males (postcopulatory or cryptic female choice; Thornhill 1983; Eberhard 1996; Pitnick and Brown 2000), received growing attention (e.g. Birkhead 1998; Birkhead and Pizzari 2002). In particular, the potential ability of females to discriminate and differentially utilize sperm of different males within their reproductive tract, so called sperm selection or "sperm choice" (Birkhead 1998), became focus of an intense scientific debate (Birkhead 2000; Eberhard 2000; Kempenaers et al. 2000; Pitnick and Brown 2000).

One main potential benefit to females of cryptic sperm selection is to increase the genetic quality of their offspring. Females could achieve this by selecting for particular "good genes" or by selecting for a genetically more compatible genome (for review see Zeh and Zeh 1996; Jennions and Petrie 2000; Tregenza and Wedell 2000). At the interspecific level numerous studies showed that conspecific sperm have a higher probability of fertilizing an egg than heterospecific sperm (Dziuk 1996; Jennions and Petrie 2000). Similarly, sperm from a male from the same race, population, or strain often have a higher fertilization capacity (Markow 1997; Brown and Eady 2001). On the other hand, inbreeding has negative fitness consequences (reviewed in Pusey and Wolf 1996; Keller and Waller 2002), whereas increased individual heterozygosity has positive fitness consequences (e.g. Amos et al. 2001; Foerster et al. 2003). Sperm selection may thus be driven by the costs associated with inbreeding and outbreeding. Selection of sperm based on sperm genotype could be a mechanism to select the genetically most compatible sperm, not only after copulating with two or more conspecific males (e.g. Bretman et al. 2004), but even within a male's ejaculate (Marshall et al. 2003). In the following we will

focus on postcopulatory female sperm selection within the female reproductive tract only based on sperm genotype.

Growing support for female sperm choice based on male genotype comes mainly from studies on invertebrates (Bishop 1996), in particular insects (e.g. Wilson et al. 1997; Clark et al. 1999; Stockley 1999; Mack et al. 2002; Nilsson et al. 2003; Bretman et al. 2004). However, much of the evidence remains suggestive rather than conclusive, because all studies are based on natural matings. In this situation it is not possible to distinguish between differential sperm numbers inseminated, differential sperm uptake during copulation and sperm selection within the female reproductive tract after copulation (e.g. Mack et al. 2002, Nilsson et al. 2003, Bretman et al. 2004).

In vertebrates, the evidence for female sperm selection based on the genotype of conspecific sperm is also inconclusive. Two studies in the guppy (*Poecilia reticulata*) showed directional postcopulatory sexual selection for more colourful males (Evans et al. 2003; Pilastro et al. 2004). However, superior fertilization success of more colorful males can be the result of such males transferring ejaculates of superior quality (Evans et al. 2003), and/or females accepting more sperm from more colorful males (Pilastro et al. 2004). Whether there is further sperm selection within the female reproductive tract cannot be confirmed nor rejected by these studies. A similar problem applies to studies on sand lizards *Lacerta agilis* (Olsson et al. 1996; Olsson et al. 1997; Olsson et al. 2004), which show that genetic relatedness explains a significant part of the variation in fertilization success under sperm competition. However, this effect can also be attributed to unrelated males transferring more sperm, respectively females accepting more sperm from unrelated males. An experimental study in the domestic fowl (*Gallus gallus domesticus*) found that paternity success varied across females, which were inseminated with equal numbers of sperm from two males (Birkhead et al. 2004). It remained unclear whether this is the result of cryptic sperm selection or early embryo mortality. Recently Pizzari et al. (2004) demonstrated that female red junglefowl (*Gallus gallus*) retained fewer sperm following natural inseminations by brothers, despite the fact that in a second experiment male fowl were found to inseminate even more sperm into sisters than into unrelated females. Again it remains unclear whether females differentially ejected sperm or whether sperm selection took place within the female reproductive tract. Support for

sperm selection at the level of the egg stems from an in-vitro fertilization experiment with mice (Rülicke et al. 1998). This study observed non-random fertilization with respect to MHC haplotype. Although there is some further evidence of selective immunological reactions against sperm inside the mammalian reproductive tract (Cohen and Werrett 1975; Dondero et al. 1978; review in Zeh and Zeh 1997), several other studies failed to detect any effect of female sperm selection (common shrew: Stockley 1997; mallard: Cunningham and Cheng 1999; domestic fowl: Birkhead et al. 1999).

The above suggests that the mixed evidence for cryptic sperm selection might partly stem from the difficulties to disentangle female-mediated effects on the outcome of paternity from biases caused by sperm competition (Birkhead 1998; Birkhead 2000; Eberhard 2000; Kempenaers et al. 2000; Pitnick and Brown 2000). Sperm competition and cryptic female choice are two processes that occur simultaneously. When a female copulates with several males, sperm from these males will compete inside the female's reproductive tract to fertilize her ova (Parker 1970). Thus, any observed bias in paternity may be purely male-mediated due to differences in the amount of transferred sperm (Cook and Wedell 1996), variation in sperm quality (e.g. mobility, Birkhead et al. 1999) or size (Radwan 1996), or mating order (Birkhead and Parker 1997). To clearly demonstrate female sperm selection within the female reproductive tract it is essential to control for these effects. Earlier studies based on natural matings did not control for the numbers of transferred sperm (e.g. Olsson et al. 1996; Stockley 1997; Wilson et al. 1997; Clark et al. 1999; Mack et al. 2002; Nilsson et al. 2003) or did so only in indirect ways via male size and age (Stockley 1999), or via the presence of a spermatophore (Bretman et al. 2004). No information about individual male sperm quality was available in any of these studies.

We studied the relative importance of cryptic female choice and sperm competition in mallards (*Anas platyrhynchos*). Species like mallards are of particular interest for such studies, because females frequently lack behavioral (precopulatory) mechanisms to control the transfer of sperm. Mallards form socially monogamous pairs in autumn and it is assumed that females base their mate choice predominantly on indirect (genetic) benefits since drakes do not provide obvious direct benefits such as territories or help with brood care (Cunningham 1997). Although female mallards show

strong preference for their social partner (Bluhm and Gowaty 2004) and apparently do not incite, but strongly resist extra-pair copulations (Cunningham 2003), they commonly suffer from coerced copulations by other males (Cunningham 1997; Davis 2002). However, copulations with nonpreferred males lead to significant fitness reduction for female mallards (decreased offspring viability and mother productivity, Bluhm and Gowaty 2004). Unlike most bird species, drakes possess a penis-like intromittent organ, which allows males to deposit the ejaculate deep inside the female's reproductive tract. This should further reduce female behavioral control, because sperm ejection (Pizzari and Birkhead 2000) might be less likely. Therefore ducks might have evolved other postcopulatory mechanisms to assure fertilization by the preferred male.

In a previous study of sperm selection in mallards, Cunningham and Cheng (1999) artificially inseminated ducks with a mixture of sperm from males of two different genotypes (white plumage and wild type). This study failed to detect consistent sperm use between inseminations and therefore dismissed cryptic sperm selection by female mallards purely based on genotype. However, in Cunningham and Cheng's (1999) study, sperm of eight different males per genotype was pooled for insemination and therefore females were inseminated with a mixture of sperm from 16 different males. No information on the representation of each single male in the insemination mixture or about sperm quality was available. Because both sperm density (numbers per unit of ejaculate volume) and quality can vary dramatically between ejaculates in this species (C. Stunden, pers. comm. 2001; this study), variation in composition and quality of the used sperm mixture might have obscured effects of female sperm choice.

The aim of our study was to investigate the relative importance of sperm characteristics and cryptic female sperm selection in determining paternity. Here we present the results of an experiment where female mallards were artificially inseminated with a sperm mixture containing equal sperm numbers from one brother and from one unrelated male. This method allowed us to rule out effects of mating order, to control for the number of transferred sperm, and to measure the quality of the sperm from each male. We used sperm from a brother and an unrelated male, because matings between siblings represent an extreme case of inbreeding and reduction in heterozygosity. If mechanisms

to avoid inbreeding or to increase offspring heterozygosity have evolved, we expected them to become apparent in such an extreme case.

Our experimental procedure allows us to make the following predictions. (1) If fertilization success is solely based on the number of transferred sperm, and neither sperm quality nor cryptic female sperm choice influences the outcome, both competing males should gain equal amounts of paternity. (2) If sperm quality determines fertilization success, the male with the highest sperm quality should gain most paternity, independent of his relatedness to the female. (3) If sperm selection enables female mallards to discriminate against sperm of closely related males in order to avoid negative effects of inbreeding, the unrelated male should gain most paternity.

MATERIAL AND METHODS

Animals

To establish families of known relatedness, nine pairs of free-living mallards were caught in March 2000 at different locations around two lakes ('Starnberger See' 47º54'N/11º18'E and 'Ammersee' 48º00'N/11º08'E) in Southern Germany. Pairs were kept in separate aviaries and allowed to breed for three months. All first and second clutches were collected and artificially incubated. Because only five pairs produced large enough families for our purposes (at least four females and one male), four more experimental groups were raised in 2001 from mallards of the smaller families and unrelated wild-caught ducks. Parentage of all ducklings was confirmed by microsatellite analysis (see below).

Ducks were kept in two 10 x 6 m outdoor aviaries with a 4 m^2 concrete pond each, and three outdoor aviaries (15 x 9, 15 x 9 and 15 x 11 m) situated at the shore of a small lake with two thirds of the aviaries' area covered by lake water. During the experiments, the three large aviaries were divided to create a total of seven separate compartments. We provided the birds daily with commercial duck food (Anseres 3, Kasper Faunafood, Woerden, Netherlands) mixed with wheat, except during the breeding season, when we provided special breeding pellets (Anseres 4, Kasper Faunafood, Woerden, Netherlands). The birds received fresh lettuce at least three times a week.

Experimental Design

We used a sperm mixture containing equal numbers of sperm from two unrelated males (sperm donors) to artificially inseminate four sisters of each male, that is, at each insemination eight females were inseminated with the same sperm mixture. We conducted the experiments with 12 groups of four sisters, from a total of nine genetically unrelated families. In total we had six different male pairings whereby three males were used twice in two different combinations. We performed repeated inseminations using the same individuals (sisters and male pairs) in identical combinations throughout the study.

Between April and June 2002 sperm collection and inseminations took place once a week and inseminations were conducted when viable sperm of both males could be collected (at least 30% of sperm cells motile after sperm collection). In total we performed 224 inseminations on the 48 individual females, over a period of eight weeks (4.7 inseminations per female, range: 3-7; se ± 0.7). For practical reasons inseminations were conducted for all females on the same dates and could not be matched with individual egg-laying cycles.

During the experiments females were kept isolated from males. Because aviary space was limited and ducks could not be housed individually we kept females in groups of four sisters. We kept these groups either separately or together with another, genetically distinct family, which was not inseminated by the same pair of males, or together with ducks which were not inseminated at all (16 ducks for potential replacement). We provided each aviary with (number of ducks + 2) nesting boxes and with nesting material (straw).

Semen Collection and Artificial Insemination (AI)

Sperm donors were kept isolated in small aviaries (2.4-6.1 m^2, including a water tub) together with one female from the end of January 2002 onwards. From February until early June males were exposed to the sperm collection procedure (see below) at least once a week. To prevent copulation shortly before sperm collection the female partners were removed the day before each collection and returned afterwards. To increase sperm quality and quantity females were removed completely from the end of April onwards and the males were kept singly. On the evening before the day of sperm collection, food

was removed from the aviaries to minimize fecal contamination of ejaculates. Food was replaced immediately after sperm collection.

Sperm were obtained following the massage procedure described in Lake and Stewart (1978). We modified this method in so far that we collected the ejaculate directly in a 1 ml syringe (no needle) placed at the base of the intromittent organ, where the ejaculate emerges before running along an open drain to the tip of the intromittent organ. Sperm samples of the pair of sperm donors were always taken within 30 min (maximum) and were inseminated within 1 hr after collection. On average we obtained an ejaculate size of $12.7*10^7$ spermatozoa (range: $3.6*10^7$-$47*10^7$; se ± $1.9*10^7$; ejaculate volume: 50-200 µl), which is comparable to ejaculate sizes collected by the massage procedure in other studies of mallards (between $5.3*10^7$ and $10.6*10^7$ spermatozoa in yearling and adult drakes respectively; Stunden et al. 1998). To prevent sperm dehydration and to facilitate insemination of accurate volumes (and hence sperm numbers) we used Ringer-Lactate as sperm extender, which is suggested as avian sperm diluent by Smyth (1968) and has shown to effectively preserve mallard sperm motility over several hours (Humphrey 1972; pers. observation). Immediately after collection, the ejaculate was diluted circa 1:3 with Ringer-Lactate solution (Ringer-Lactat after Hartmann, Z.Nr. 1-19.566, Mayrhofer Pharmazeutika GmbH, 4020 Linz, Austria) and this suspension was used in further procedures.

The concentration of sperm in the sperm suspension was determined in three counts of two dilutions in an improved Neubauer counting chamber (repeatability of counts within ejaculates: $r=0.925$, $n_0=6$, $p<0.001$, N=56 ejaculates; Lessells and Boag 1987). Immediately prior to insemination we mixed the sperm suspensions so that one insemination volume contained equal numbers of spermatozoa from the two males. The required volume of each sperm suspension was taken using a pipette (with a 1 ml tip, cut to widen the opening) and the mixture was gently vortexed. Ducks were inseminated with the maximum number of sperm available per male combination, on average $3.18*10^7$ sperm (range: $3*10^6$ – $1.2*10^8$) diluted in 200 µl Ringer-Lactate. Using an 1 ml syringe the sperm mixture was applied approx. 8-10 cm inside the female reproductive tract (Knoll 1978).

Sperm measurements

Sperm quality was analyzed using a Hobson Sperm Tracker (Hobson Tracking Systems Ltd, Sheffield, UK). We followed the protocol described by Froman and Feltmann (2000) using a blood-sperm suspension to keep sperm motile in the sperm swimming chamber over the observation period. Blood (100µl) was obtained from a non-experimental, unrelated female mallard (a different female for each insemination) before sperm collection and was diluted in 900 µl TES (50 mM N-tris-[hydroxymethyl]methyl-2-amino-ethanesulfonic acid; Sigma Chemical Co., St. Louis, MO, USA), pH 7.4, containing 128 mM NaCl and 2 mM $CaCl_2$ (TES-buffered saline)). However we found that substances provided with the blood plasma were sufficient to keep sperm activity (proportion of motile cells, sperm swimming speed) constant and an erythrocyte layer was not necessary (Denk and Kempenaers MS). To avoid mechanical and visual interference during sperm swimming measurements, blood cells were allowed to settle at the bottom of an Eppendorf-tube and only the clear phase of the blood-TES-buffered saline suspension was used in the sperm swimming chamber. The ejaculates in Ringer-Lactate solution (see above) were further diluted 10:1 with this blood-TES-buffered saline solution to a final concentration of 3 million cells per ml.

We injected the sperm suspension in a pre-heated (38°C) MicroCell swimming chamber (50 µm depth; Conception Technologies, San Diego, USA). All observations on sperm motility were made at 38 °C. We chose this temperature slightly below avian body temperature to reduce effects of evaporation on sperm swimming behavior and to make long-term observations possible. We used a microscope with a 4 x bright-field objective under pseudo dark-field conditions using a Ph3 annular phase ring, and a total magnification of 48. The sperm were videotaped for 15 min after injection in the sperm swimming chamber, using a Sony SPT-M128 CE Black&White video camera and a Panasonic (NV-HS 900) Super VHS video recorder. For each ejaculate three replicates were recorded. With the ‘minimum track time’ set at 1.2 sec for individual sperm continuous observations (Froman and Feltmann 2000), we used the Hobson Sperm Tracker to measure straight-line velocity (VSL) and the number of tracks, a measure of the percentage of motile sperm in the final concentration of 3 Mio cells/ml, at four time intervals: 0-2 min, 4-6 min, 8-10 min and 12-14 min.

To obtain another, independent measure of sperm quality, sperm motility was also estimated visually as follows. Five minutes and 2 - 2 ½ h (identical times for both ejaculates in each pair) after dilution we placed a drop of sperm suspension (diluted as described above for the Hobson sperm tracker) in an improved Neubauer Counting chamber and kept it at 38 °C. We videotaped the sample under 25 times magnification (phase-contrast ring 100) using an Olympus BH-2 microscope under standard bright-field conditions. Per ejaculate we filmed three replicates for one minute each, using a Teli CCD color camera (CS5130OP) and a Panasonic (NV-HS 900) Super VHS video recorder. Two people independently assessed the percentage of motile sperm (0-100%, in steps of 10%). Values obtained by the two observers were highly repeatable (repeatability $r=0.94$, $N=291$ assessments, $p<0.0001$). This easy and reliable technique is also commonly used in AI for commercial breeding (e.g. Knoll 1978).

To measure sperm length, we stored samples from each ejaculate in formaldehyde (10%; Roti®-Histofix, Carl Roth GmbH, Karlsruhe, Germany). A drop of this solution was placed on a slide and observed under an Olympus BH-2 microscope at 200x magnification (phase-contrast ring 40). We determined the overall length of sperm using the analytical imaging software Optimas 6.5 (Media Cybernetics, Silver Spring, USA). Three slides were made for each ejaculate and 20 sperm per slide were measured. Abnormal sperm (e.g. bent heads, heads without tails) were not found.

Fertilization success

Eggs were collected every second day and transferred to an incubator (computer-assisted motor-incubator SV250, J. Hemel Brutgeräte, Verl-Kaunitz, Germany). After three days of incubation, embryo development was checked by candling with an ORBAN candling lamp (Tempo Nr. 119). Developing eggs (embryo visible as a disc) were opened and the embryo was stored in 70% ethanol. Of 114 fertilized and developing eggs, three contained visibly dead embryos and two further embryos showed disturbed embryonic development (defunct blood vessels) when opened at day three. Four of these five unsuccessful embryos were sired by a brother of the focal female. Undeveloped eggs were left in the incubator for another three days and were then opened to look for signs of development or early embryonic death (e.g. circular contracted blood vessels); none of

these eggs showed such signs. Maternity of the fertilized eggs was confirmed by microsatellite analysis (see below) and showed that they belonged to 36 different females, in 'clutches' of up to four eggs following an AI event. This clutch size is much lower than a normal clutch size for mallard females because laying cycles of females were not matched to insemination events (that is, part of the 'clutch' was laid before insemination).

Due to space limitations, we had to keep additional females, which were not inseminated, in the same aviaries with the focal females. Because ducks sometimes share nests or lay their eggs in other nests, undeveloped eggs could not be unambiguously attributed to individual females. Molecular assignment of maternity of undeveloped eggs on the basis of genetic markers is difficult (e.g. Arnold et al. 2003). Therefore we had to estimate fertilization success of our AI method as follows: first we took into account that ducks require AI every 4-5 days to keep producing fertile eggs (Smyth 1968; Lake and Stewart 1978). Thus, our estimate of fertilization success is based on all eggs laid up to four days after an insemination event. We also had to consider the presence of unfertilized eggs laid by non-focal females. Therefore fertilization success after AI was estimated from the total number of eggs laid in an aviary during the first four days after an insemination event multiplied by the proportion of ducks inseminated in an aviary. This gave the number of eggs, which could potentially have been fertilized (N = 294) and was used to estimate fertilization success. On average 48% of eggs laid after an insemination event were fertilized (range: 13 % - 100 %, see Figure 1). Because ducks laid eggs throughout the day, some inseminations took place after the period when the next day's egg could have been fertilized and our calculations may somewhat underestimate true fertilization success.

Compared to fertilization success in clutches of free-living mallards (96%; pers. observation) this fertilization success of AI is rather low, but comparable to other AI studies. In mallards, fertilization success of 70 % has previously been achieved (Stunden et al. 1998), while Cunningham and Cheng (1997) reported complete failure of 11.4% of clutches. In Moscovy ducks (*Cairina moschata*; Knoll 1978), 66% fertilization success was achieved. However these studies used twice (Stunden et al. 1998), five times (Knoll 1978) and twelve times (Cunningham and Cheng 1997) as much sperm as we did. Beside

effects of AI, reduced fertilization might be a result of sperm depletion in our study. In our experiment, females were inseminated once a week with only a fourth of a male's ejaculate, whereas free-living female mallards copulate on average twice a day (Cunningham 1997). Indeed, we found a positive relationship between the number of inseminated sperm and the percentage of fertilized eggs (Fig 1; GLMM: response variate: number of fertilized eggs on four days post insemination; binomial denominator: number of eggs produced by a group of females; explanatory variate: number of sperm inseminated; random factor: experimental group; Wald F=3.21, df=1, p=0.073). Thus, the number of sperm used in the AI might have been at the lower limit necessary for successful fertilization. Although it is possible that females do not select sperm to assure fertilization when sperm is limited, it seems unlikely that a sophisticated mechanism of sperm selection based on the amount of available sperm has evolved in a species where females are unlikely to ever be sperm restricted. Therefore, we fail to see a theoretical reason why the mechanism of female sperm selection would not operate with the sperm numbers used here.

To verify whether females suffered from a lack of sperm we inspected the germinal disc of five eggs for signs of early development (Kosin 1944; Kosin 1945), which we failed to find. To exclude ambiguities we further examined the perivitelline membranes in a random sample of 20 undeveloped eggs from clutches which contained at least one fertilized egg, following the method described by Birkhead et al. (1994). Sperm were stained with the fluorescent Hoechst dye 33258. We searched for trapped sperm at three different spots of the perivitelline membrane (at 200x magnification), checking 5-10 % of the total membrane area. Previous observations on naturally fertilized eggs suggested that sperm are evenly distributed over the entire membrane (Wishart and Staines 1999). Furthermore, we counted the sperm on twelve naturally fertilized mallard eggs (from four broods) and found a higher sperm density on areas other than the blastodisc region (mean number of sperm / mm^2 ± s.e.: blastodisc: 19±4.8; other region: 32±7.7; REML analysis: response variate: number of sperm per mm^2; fixed factor: membrane area (blastodisc or other region), Wald F=6.69, df=1, p=0.01; random factors: duck identity, p=0.041; egg identity nested in duck identity, p=0.23). In the present study no sperm cells were detected on any of the 20 experimental eggs. Because the probability

of fertilization is correlated with the number of sperm found on the membrane (Wishart and Staines 1999), our findings indeed suggest that the eggs failed to develop because of a lack of sperm, not because of early embryonic death.

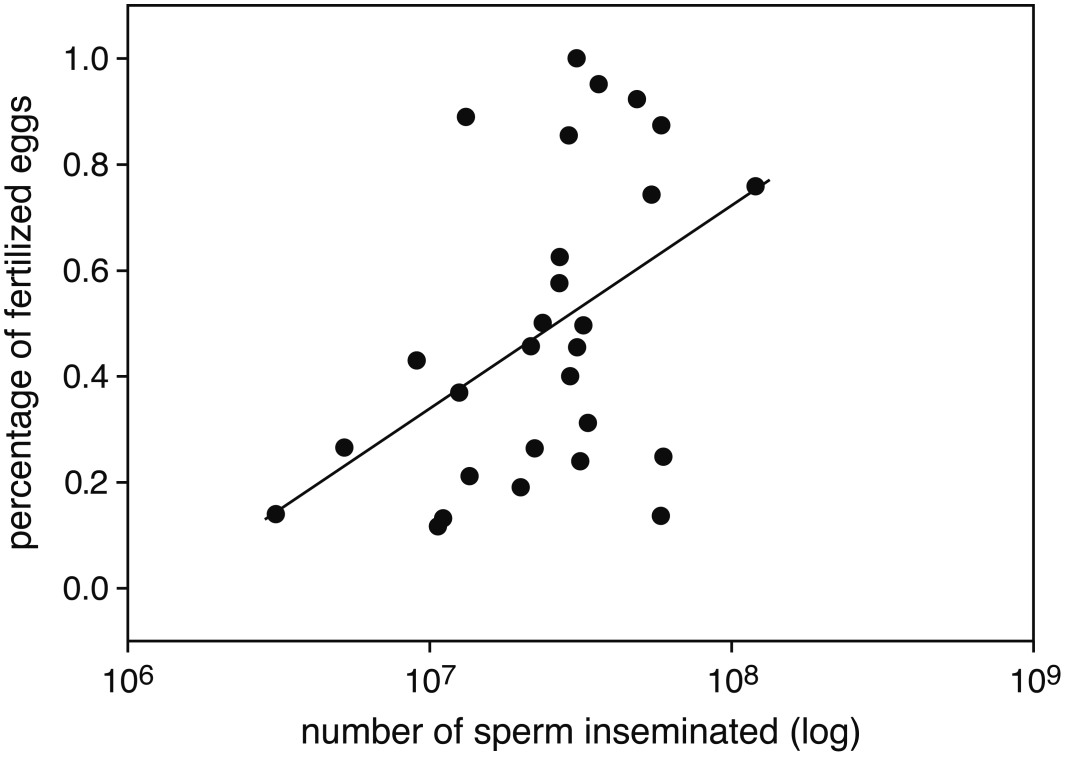


Figure 1. Fertilization success in relation to the number of sperm artificially inseminated (Spearman rank correlation: r=0.42, p=0.028; N=28 insemination events of eight females each). Note the logarithmic scale. The solid line represents the regression line. See Methods for further details.

Parentage analysis

To extract DNA from embryos we used DNeasy® Tissue Kit (Qiagen). Paternity was assigned by using seven polymorphic microsatellite-markers (*APL 2, APL 11, APL 12, APL 14, APL 23, APL 26, APL 36*; see Denk et al. 2004). In the nine unrelated males used in this study, we found six to 11 alleles per locus, leading to a combined exclusion probability of more than 99.98% (Jamieson and Taylor 1997). We followed a standard PCR protocol (10 µl reaction) using 1 µl genomic DNA, 100 µm dNTP, 0.12 µl of a 1% Bovine Serum Albumen, 1.5 mM MgCl2, 10 mM 10x Mg-free buffer (Promega) and 0.25 U of Taq DNA polymerase (Promega). In the PCR profile 60 °C was used as annealing temperature. Amplified fragments were resolved on an ABI Prism 310 Genetic Analyzer (Applied Biosystems). All offspring alleles were unequivocally assigned to one of the females and one of the two males used in an insemination.

Statistical Analyses

We calculated the repeatability (Lessells and Boag 1987) of measurements of sperm characteristics both within an ejaculate (measurement repeatability) and between ejaculates (seasonal repeatability, Table 1). All measurements of sperm characteristics made within the same ejaculate were highly repeatable ($r=0.59-0.85$, all $p<0.001$), suggesting that our methods were reliable and measurement error was low.

		Seasonal repeatability		
Sperm characteristics	Statistic	r	n_o	p
Size	$F_{8,47}$=20.45	0.76	6.17	<0.001
Swimming				
VSL (0-2 min)	$F_{8,48}$=2.34	0.18	6.26	0.033
VSL (4-6 min)	$F_{8,47}$=2.21	0.17	6.16	0.044
VSL (8-10 min)	$F_{8,42}$=2.48	0.21	5.70	0.027
VSL (12-14 min)	$F_{8,40}$=2.06	0.16	5.39	0.064
Track (0-2 min)	$F_{8,48}$=1.22	0.05	6.26	0.27
Track (4-6 min)	$F_{8,47}$=0.84	-0.03	6.16	0.57
Track (8-10 min)	$F_{8,42}$=1.21	0.04	5.70	0.32
Track (12-14 min)	$F_{8,40}$=1.42	0.07	5.39	0.22
Overall motility (5 min)	$F_{8,20}$=2.10	0.26	3.12	0.085
Overall motility (2 h)	$F_{8,44}$=2.01	0.15	5.98	0.067

Table 1. Repeatability of sperm measurements within males across ejaculates (seasonal repeatability). n_0: average number of repeated measures. VSL: straight-line velocity, Track: number of tracks recorded in 2 min, Motility: percentage motile cells as scored by eye (see Methods for details).

We did not find significant correlations between sperm size and the other measures of sperm quality (REML controlling for male identity as a random factor, see below statistical analyses: all $p > 0.1$, except for VSL after 0-2 min: Wald F=3.17, df=1, p = 0.075; motility after two hours: Wald F=3.05, p=0.081). Thus, in all models, sperm size and swimming performance were considered independent variables.

Since we performed repeated inseminations of related females our data was structured, with repetition at three levels, the level of the female (repeated insemination of each female), the family (related females) and pairs of males used for repeated insemination. Such structured data are best analyzed using mixed models, where the random term specifically models the pseudoreplication associated with the structure (Grafen and Hails 2002, chapter 12). Our data set was not orthogonal due to unequal sample size precluding the use of ANOVA. Therefore, for normally distributed dependent variables we used restricted maximum likelihood (REML) mixed models, while for analyses of proportions we used generalized linear mixed models (GLMM) with a binomial error.

To analyze changes in sperm swimming traits over the 14 minutes of observation we used REML mixed models controlling for multiple ejaculates per male by including male identity as a random factor in the model. We compared the number of observed tracks and sperm swimming speed (VSL) at each of two consecutive time intervals (see Fig. 2).

To test the effects of relatedness and sperm quality on fertilization success we used GLMM models with binomial error using ‘number of eggs sired by male A’ as response variable and ‘clutch size’ (all fertilized eggs laid following a single insemination) as the binomial denominator. In these models, we accounted for repeated inseminations of females with sperm from the same pair of males and for relatedness of groups of inseminated females by including ‘female id’, ‘family’ and ‘male pair’ as random effects in the models. Effects of these random factors were not strong. Random effects ‘female id’ and ‘family’ were not significant (all $p > 0.70$). Some evidence of significant effect was observed for ‘male pairs’ ($p=0.3$-0.02). Inclusion or exclusion of the random factors made no qualitative differences in the results. Also inclusion of the term ’insemination event‘ as a random factor to control for differences among insemination events did not change the conclusions.

In these GLMM models analyzing fertilization success, one of the males represented in each sperm mixture was randomly (male with the lower leg band number) assigned male A and his gain of paternity (proportion of eggs sired) was analyzed (as in Evans et al. 2003). In all analyses, the difference in sperm quality between male A and

male B was used as explanatory variable. Relatedness, sperm size and one measure of sperm quality (either number of tracks, VSL, or percentage motile cells) were fitted as fixed effects (explanatory variates) to each model. Sperm quality measurements made at different time intervals (see above) were analyzed in separate models.

We initially constructed full models containing all explanatory variables. Non-significant terms were dropped from the model until the final model only contained variables with $p < 0.10$. All eliminated terms were then re-added to the final model to confirm their lack of significance and these p-values are reported here. We included the interaction term 'relatedness' x 'sperm quality measure' in the model to test the hypothesis that sperm have to be of higher quality (swim faster) to be successful if the male is closely related to the female. Other interaction terms could not be tested due to low sample size. We used Genstat 6.1.0.200 (Genstat 2002) for all statistical analyses.

RESULTS

Sperm characteristics

Taking each ejaculate as a datapoint (N=56) mallard sperm showed a mean VSL (± s.e.) of 41.77 ± 1.56 μm/sec (range 4.83 – 88.20) and an overall percentage of motile sperm (± s.e.) of 48.1 % ± 3.3 (range 5 % - 88.67 %). Straight-line velocity (VSL) and number of recorded tracks varied significantly over the observation period (REML; VSL: Wald F= 8.05, df=1, $p < 0.001$; number of tracks: Wald F=6.60, df=1, $p<0.001$). VSL increased between the first two observation periods and remained at a constant level thereafter (see Fig. 2). The increase in VSL was not simply caused by a reduction in the number of recorded tracks, because the number of tracks decreased only after 6 min (see Fig. 2). The average percentage of motile sperm did not change significantly between 5 min and 2 - 2 ½ h after dilution.

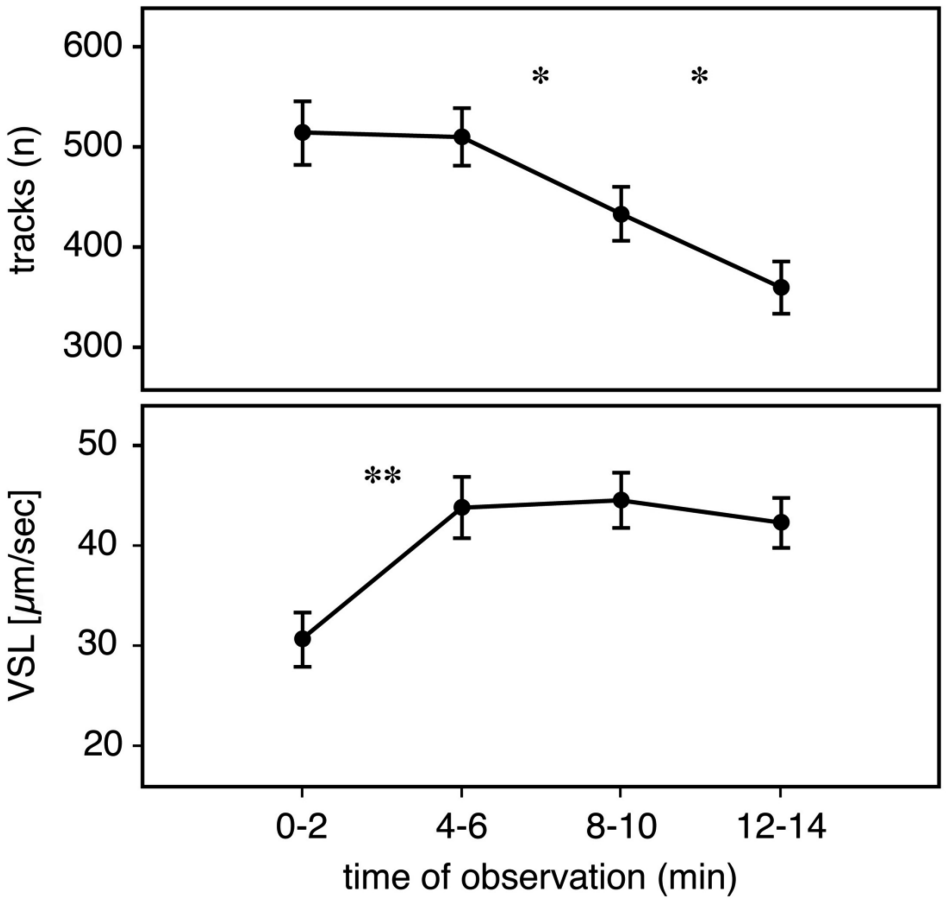


Figure 2. Change of sperm swimming behavior (number of tracks and straight-line velocity -VSL) over time (N=56 ejaculates). Changes are significant at $p < 0.05$ (*) and $p < 0.001$ (**) based on REML analyses controlling for male effects (N=9 males; see text for details). Error bars indicate s.e. values.

Over the course of the study (eight weeks) sperm size was highly repeatable among males, but the seasonal repeatability of measurements of sperm quality was much lower, and not always significant (see Table 1).

Paternity

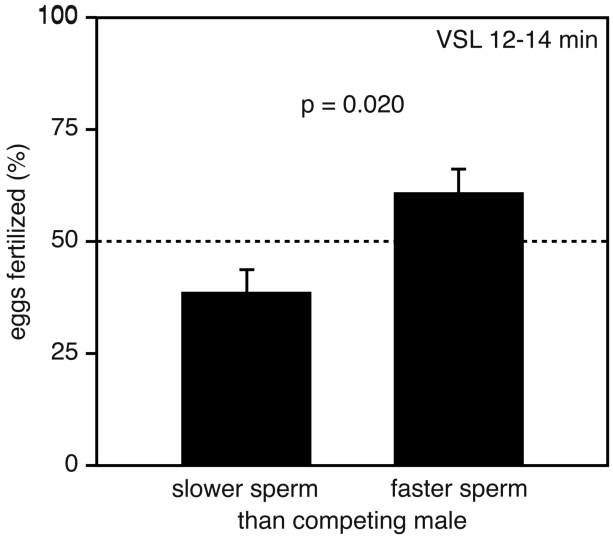
A male's fertilization success was related to long-term sperm performance. Both the number of tracks and VSL after 8 min or more significantly predicted paternity (Table 2, Fig. 3A). In general, sperm motility was the best predictor of paternity (Table 3, Fig. 3B), while sperm size did not affect fertilization success (Table 2 and 3). Given the absence of significant random effects and the large individual variation in sperm quality, we also present simple correlations between sperm quality measurements and fertility: VSL 8-10min, r=0.261, p=0.028, N=73; VSL 12-14min, r=0.297, p=0.012, N=70; motility 2 hours, r=0.373, p=0.002, N=69.

	0-2 min		4-6 min		8-10 min		12-14 min	
Independent variants	Wald	p	Wald	p	Wald	p	Wald	p
Relatedness	0.06	0.80	0.10	0.76	0.21	0.65	0.07	0.79
Sperm Size	0.64	0.42	0.75	0.39	1.57	0.21	1.68	0.19
No. of tracks	2.25	0.13	3.46	0.063	6.39	0.011	5.87	0.015
Relatedness	0.05	0.82	0.13	0.72	0.02	0.89	0.03	0.87
Sperm Size	0.52	0.47	0.56	0.45	0.60	0.44	1.19	0.28
VSL	2.48	0.12	1.91	0.17	4.55	0.033	6.18	0.013

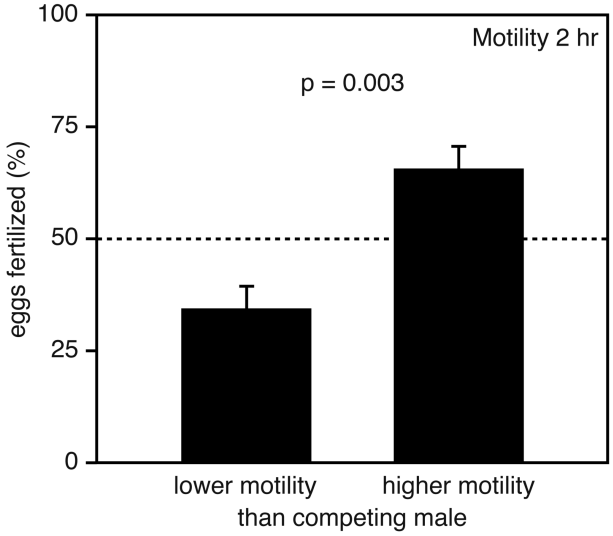
Table 2. Results from GLMM-analyses of factors determining fertilization success and correcting for repeated measures. Four separate models were constructed for each measurement time (time after injection in the sperm swimming chamber). These models were built separately for number of tracks and straight-line velocity (VSL; see Methods for details). The Wald statistic is shown, which follows a chi-square distribution with df=1.

	5 min		2h	
Independent variants	Wald F	p	Wald F	p
Relatedness	0.06	0.81	0.00	0.98
Sperm Size	0.04	0.85	0.13	0.72
Motility	0.30	0.58	8.23	0.004

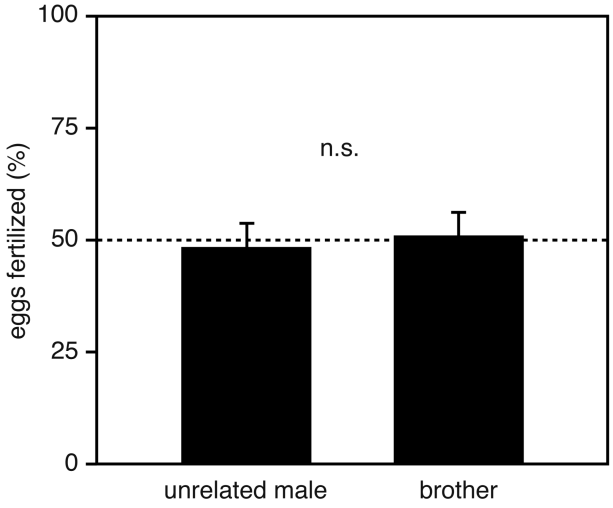
Table 3. Results from GLMM-analyses of the effects of motility (the percentage motile cells, 5 min and 2-2 ½ h after dilution with sperm extender) on fertilization success, correcting for repeated measures (see Methods for details). The Wald statistic is shown, which follows a chi-square distribution with df=1.


100
75
50
25
0
eggs fertilized (%)
VSL 12-14 min
p = 0.020
slower sperm
faster sperm
than competing male

A


100
75
50
25
0
eggs fertilized (%)
Motility 2 hr
p = 0.003
lower motility
higher motility
than competing male

B


100
75
50
25
0
eggs fertilized (%)
n.s.
unrelated male
brother

C

Figure 3

Figure 3. Mean percentage of eggs sired after a single insemination by a male, when sperm from this male showed (A) a lower or higher straight-line velocity (VSL at 12-14 min) (N=70 clutches) or (B) a higher or lower overall motility (percentage motile cells at circa 2 - 2 ½ h after dilution) than his competitor (N=69 clutches) or (C) when the male was a brother of the female or unrelated (N=73 clutches). Error bars indicate s.e. values. p-values are based on GLMMs (see text for details).

Whether or not a male was related to the female did not have any effect on fertilization success (see Table 2 and 3, Fig. 3C). This remained also true if only insemination events were analyzed in which the ejaculates from both males showed similar VSL or percentage of motile cells (maximum difference: ± 4 s.e., N=26 clutches, details not shown). Ejaculates from three males were used in two experiments (different male pairs). Reducing the dataset so that each male appeared in only one comparison did not change the significance levels despite smaller sample size (N=49 clutches, details not shown).

We did not find evidence that fertilization success depended on an interaction between sperm quality and relatedness (in all cases, $p > 0.05$).

DISCUSSION

Our experiment shows a clear effect of variation in sperm quality (swimming speed and sperm motility) on fertilization success in mallards. The degree of genetic similarity between parents did not influence paternity, when ducks were artificially inseminated with a sperm mixture of a first-degree relative and an unrelated male. This may suggest that female mallards were not able to select sperm purely based on the sperm's genotype. Furthermore, unlike a study on sedge warblers (*Acrocephalus schoenobaenus*; Marshall et al. 2003), the allele frequency of the successful sperm did not deviate from a distribution expected by chance ($p = 0.29$, details not shown).

The results of the present study emphasize the importance of sperm quality for fertilization success (Snook 2005). In our study long-term sperm behavior was the important factor predicting paternity. The effect sizes of number of active sperm, sperm

swimming speed and overall motility increased over time and only became significant after eight or more minutes. Birkhead et al. (1999) also found that sperm quality determined paternity under sperm competition. They artificially inseminated domestic fowl, *Gallus gallus*, with a sperm mixture containing ejaculates from one male with low- and one with high-mobility sperm. Males with high-mobility sperm fathered the majority of offspring. Birkhead et al. (1999) found no female effect on variation in paternity, highlighting the importance of sperm competition for fertilization success.

Although studies on insects and lizards suggest that females might be capable of sperm selection based on compatible sperm genotypes (Olsson et al. 1996; Wilson et al. 1997; Clark et al. 1999; Stockley 1999; Mack et al. 2002; Nilsson et al. 2003; Bretman et al. 2004), our findings corroborate the conclusions from previous studies on birds and mammals (mallards: Cunningham and Cheng 1999; common shrew *Sorex araneus*: Stockley 1997). Even though a detailed assessment of sperm quality was missing in the latter two studies, no effect of sperm selection was detected, and fertilization success could be explained in terms of sperm competition.

Even in the absence of female sperm selection, sperm competition *per se* might be a mechanism by which females select the “best” male. If ejaculate quality reflects male quality, females might have the majority of their offspring sired by the high quality males. However, there is little evidence that genetic quality translates into sperm quality. In a mammal (lion, *Panthera leo*) genetic quality (individual genetic diversity) influenced sperm quality (e.g. incidence of abnormal sperm; Wildt et al. 1987). In mallards, sperm swimming speed (VSL at 8 min or more) correlated with a measure of the carotenoid-based beak color (Peters et al. 2004), a sexually selected trait in this species (Omland 1996a; Omland 1996b). However, other studies failed to find a correlation between preferred male phenotypic traits and sperm quality in birds (Birkhead and Fletcher 1995; Birkhead and Petrie 1995; Birkhead et al. 1997; Pizzari et al. 2004).

By ensuring fertilization by the faster and more motile sperm, females might also increase the chances that their sons will produce competitive sperm, if sperm quality is a heritable trait passed on by the father (sexually selected sperm hypothesis; Keller and Reeve 1995). An experimental study on fowl, *Gallus gallus domesticus*, showed that sperm mobility was highly heritable (Froman et al. 2002). However, the study also

suggested that sperm mobility may be largely under the control of an independent maternally inherited element (Froman et al. 2002). In that case, fertilization by the faster sperm would not influence the competitive ability of sons. To our knowledge nothing is known about the heritability of sperm quality in mallards.

During our study, sperm quality (sperm swimming speed and motility) showed high variability within individual drakes (see Table 1), in agreement with observations in passerine species (Birkhead and Fletcher 1995) but in contrast to domestic fowl (Froman and Feltmann 2000; Froman et al. 1999). Variation in sperm quality between ejaculates might stem from difficulties to collect sperm samples from drakes, related to their penis-like intromittent organ. Fecal contamination (although not visible in the samples used in our experiments) or excess lymph fluid might affect sperm behavior (pers. observation). An alternative explanation for variability in sperm quality is that it reflects natural fluctuations. Regardless, the artificial or natural fluctuation of sperm quality is not expected to affect the results of our study because each insemination event was analyzed separately. However it emphasizes the importance of careful measurement of sperm quality in experiments on cryptic female sperm choice.

It is still possible that cryptic female sperm selection occurs in mallards, but that we were unable to demonstrate it, for the following reasons. First, one could argue that our experiment lacked the power to detect sperm selection because of the low sample size. This might be the case, but (a) the data do not show the slightest tendency for sperm selection based on relatedness (see Fig. 3C), and (b) despite the limited sample size, significant effects of sperm quality were found (see Fig. 3A, B). Thus, our results at least indicate that within the female reproductive tract sperm competition plays a much more important role in determining fertilization success than postcopulatory sperm selection by females. Second, a general problem with experiments such as ours arises if a bias in paternity due to early embryo mortality is erroneously interpreted as cryptic female choice. However, the results of our study are unlikely to be an effect of cryptic female choice favoring the unrelated male, unless early embryo mortality was more frequent when the unrelated male fertilized the egg and went undetected. Only this unlikely scenario would result in apparent equal paternity of the related and unrelated male. Third, it is possible that cryptic female choice occurs after natural copulations, but not after AI.

Maybe one or more aspects intrinsic to the AI technique (contamination of the sample, buffer properties, storage) compromise the sperm in such a way that cryptic female choice can no longer operate at the level of the sperm. Furthermore, during natural copulations females might obtain additional information about their partners, which might affect the success of different ejaculates. For example, Pizzari (2000) showed that female feral fowl can control paternity by selectively ejecting sperm from non-preferred males. Because we used a sperm mixture in our experiment, female mallards lacked the possibility to selectively reject sperm. Whether sperm ejection is possible in species such as mallards, where males have an intromittent organ and deposit the ejaculate further into the female reproductive tract, is unknown. If sperm ejection occurs after forced (artificial) insemination, it could account for the low fertilization success in this study. In chicken and turkeys females eject 80-90% of (artificially) inseminated sperm (Birkhead et al. 1993 and citations therein). Moreover, natural copulations may result in the sequential filling of sperm storage tubules (Briskie 1996; King et al. 2002). Segregation of ejaculates from different males within the female reproductive tract might be an important prerequisite to selectively use sperm. Inseminations with mixed ejaculates would prevent the operation of such a mechanism. Nonetheless, despite these problems, AI is the only way to experimentally control for male adjustment of ejaculate size, for example in response to the risk of sperm competition (Cook and Wedell 1996; Pizzari et al. 2003), or in response to female traits like age, body size or reproductive investment (e.g. Wedell 1992; Shapiro et al. 1994; Cook and Gage 1995; Pizzari et al. 2003).

In conclusion, we found that long-term sperm performance is an important determinant of fertilization success under direct sperm competition in this wild bird species. Although it is likely that offspring sired by a close relative suffer a higher risk of embryonic death, we did not observe evidence that sperm selection purely based on sperm genotype occurs within the female reproductive tract. Whether the success of faster and more motile sperm is a mechanism by which females ensure fertilization by the “best” male (female sperm selection favoring males producing faster sperm), or whether males are ahead in the intersexual conflict over fertilization, and female mallards have not yet developed effective mechanisms to resist forced copulations, needs further investigation.

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CHAPTER THREE

SPERM MOTILITY IN MALLARDS IS INFLUENCED BY THE FEMALE ENVIRONMENT

Angelika G. Denk & Bart Kempenaers

ABSTRACT

Sperm motility is an important determinant of male fertilization success. Recent studies in various species have shown that substances present in the ovarian fluid function in sperm attraction or activation. However, it remains untested whether there is a general stimulating effect of female substances or whether such influence is individual or context-specific. If the latter is true, this mechanism might facilitate female sperm selection. We used a computer-assisted sperm motion analysis system to investigate sperm performance (proportion of active sperm and swimming speed) of individual male mallards in different female environments. For practical reasons we exposed sperm to female blood plasma instead of ovarian fluid. Female blood plasma generally strongly stimulated sperm activity and the magnitude of this effect was female-specific. Although sperm performance was not differentially affected by blood plasma from a sister or an unrelated duck, it did differ depending on the reproductive status of the female. Sperm swam significantly faster in plasma from non-incubating (presumably fertile) females than in plasma from incubating (post-fertile) females. We speculate that this differential stimulation is due to hormonal effects. Although we failed to demonstrate an influence of relatedness, our study highlights the important influence of the female environment on sperm performance.

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INTRODUCTION

Sperm quality affects male fertilization success and thus male fitness in various species (e.g. Dziuk 1996; insects: Hunter and Birkhead 2002; fish: Burness et al. 2004; mammals: Holt et al. 1997). In birds in particular, experiments have shown that sperm motility - measured as sperm swimming speed, the percentage of motile sperm or sperm mobility (net movement of sperm) - determines fertilization success, both following an insemination by a single male (Froman et al. 1999), as well as in situations where sperm from different males compete for fertilization (Birkhead et al. 1999; Donoghue et al. 1999; Denk et al. MS). Avian spermatozoa must be motile to migrate from the site of insemination (cloaca or vagina) to the area of sperm storage (uterovaginal sperm storage tubules; Bakst et al. 1994; Ashizawa et al. 2000). It is still unclear whether sperm become quiescent during storage (Ashizawa et al. 2000) or whether residence inside the storage tubules is maintained by constant sperm activity (Froman 2003). Although even immotile, dead sperm can be passively transported up the uterus to the site of fertilization (infundibulum; Allen and Grigg 1957), sperm motility is required again to successfully penetrate the egg membrane (Ashizawa et al. 2000).

In many species, sperm motility is stimulated by substances present in the ovarian fluid (e.g. human: Jeon et al. 2001; arctic charr: Turner and Montgomerie 2002; domestic hen: Ashizawa and Wishart 1992). Among the substances that have been identified to stimulate sperm activity are peptides released by the egg (e.g. sea urchins: Suzuki 1995), steroids (e.g. ascidians: Yoshida et al. 2002), and calcium (birds: reviewed by Ashizawa et al. 2000). Whereas the general stimulating effect of these substances on sperm activity is clear, virtually nothing is known about variation in sperm stimulation depending on the individual female environment.

Regulation of sperm motility might be a way by which females can determine male fertilization success after insemination (cryptic female sperm selection). Under sperm competition female identity influenced the fertilization success of a particular male in *Drosophila* (Clark, Begun and Prout 1999) and domestic fowl (Birkhead et al. 2004). It has been shown or suggested that the outcome of sperm competition depends on the level of genetic similarity between the male and the female (Olsson et al. 1996; Stockley 1999; Mack et al. 2002; Bretman et al. 2004; see also Blomqvist et al. 2002). Thus, females

might be able to select the genetically most dissimilar sperm (see Marshall et al. 2003) to avoid the negative consequences of inbreeding (Keller and Waller 2002) or to benefit from the increased heterozygosity of their offspring (e.g. Foerster et al. 2003). However, so far, the evidence for cryptic female sperm selection is mixed (Olsson et al. 1997; Wilson et al. 1997; Clark et al. 1999; Cunningham and Cheng 1999; Stockley 1999; Denk et al. MS), and potential mechanisms remain obscure (Zeh and Zeh 1997).

We used a computer-assisted sperm motion analysis system to investigate the effect of the female environment on sperm performance in mallards (*Anas platyrhynchos*). In this species, females show strong behavioral preferences for their social partner (Bluhm and Gowaty 2004), but frequently suffer from copulations forced upon them by other males (Barash 1978; Davis 2002). Females therefore lack behavioral control over insemination, but might exert influence over fertilization inside their reproductive tract.

In ducks, the female reproductive tract is displaced further inwards (compared to other birds, e.g. chicken), which makes the repeated collection of ovarian fluid without contamination and without hurting the bird difficult. However, not only ovarian fluid contains substances that influence sperm motility. Froman and Feltmann (2000) used a blood-buffer suspension to achieve high and constant swimming speed of rooster sperm (*Gallus domesticus*). They attributed the stimulating effect to the presence of erythrocytes and oxyhemoglobin. Because we observed a similar effect on sperm activity when only the clear phase of the blood-buffer suspension was used (this study), we postulate that components of the blood plasma rather than the red blood cells stimulate sperm motility (see discussion). Given the ease with which blood samples can be taken and the fact that there is exchange between the ovarian and blood system (e.g. calcium, ovarian hormones; Whittow 2000), we used blood plasma for a first preliminary test of sperm swimming performance in different female environments.

First, we investigated whether adding female blood plasma affected sperm performance. Second, we tested whether differential sperm stimulation could facilitate sperm selection of genetically dissimilar sperm, by letting sperm of eight different males swim in the blood plasma of their sister and of an unrelated female. Third, we examined whether the effect of the blood plasma on sperm performance depends on female

reproductive status (fertile or post-fertile). Finally, we tested whether there are other consistent individual female effects on sperm swimming performance.

MATERIAL AND METHODS

Animals

We used eight male and 24 female mallards that were born in captivity (2000 and 2001) from parents caught at Lake Starnberg (47º54'N/11º18'E) and Lake Ammer (48º00'N/11º08'E), Southern Germany, or Oyten/Bremen (53°04´N/8°48´E), Northern Germany. Kinship between all individuals was known and confirmed by microsatellite analysis. During autumn and winter, mallards were kept in flocks of 24-34 animals in four large outdoor aviaries (120-165 m^2) and they were allowed to choose their mate freely. From February onwards, eight pairs were selected and housed individually in small aviaries (2.4 – 6.1 m^2, including a water tub). We provided the birds daily with commercial duck food (Anseres 3 (maintenance) or Anseres 4 (breeding season, February-July), Kasper Faunafood, Woerden, Netherlands) mixed with wheat, and we added fresh lettuce at least three times a week. After sperm collection (see below) birds were rewarded with fresh mealworms.

Sperm and blood collection

Sperm was collected from mid-April until the end of May 2003, following the method described in Knoll (1978). To stimulate the male to copulate, a female other than the social partner was introduced. Immediately before intromission, drakes were removed from the female and the ejaculate was collected into a 50 ml falcon tube. Remaining ejaculate was collected from the intromittent organ using a 1ml syringe. Within five minutes after collection sperm samples were diluted circa 1:3 with Ringer-Lactate solution (Humphrey 1972; Ringer-Lactat after Hartmann, Z.Nr. 1-19.566, Mayrhofer Pharmazeutika GmbH, 4020 Linz, Austria).

Before sperm collection, 100 µl of blood was obtained from the right cutaneous ulnar vein of a female and diluted in 900 µl TES-buffered saline (50 mM N-tris-[hydroxymethyl]methyl-2-amino-ethanesulfonic acid; Sigma Chemical Co., St. Louis,

MO, USA with 128 mM NaCl and 2 mM $CaCl_2$; pH 7.4; see also Froman and Feltmann 2000). The buffer-blood suspension was stored at room temperature and blood cells were allowed to settle at the bottom of a 1.5 ml Eppendorf tube for at least 45 min. Only the clear phase of the blood-buffer suspension (blood plasma) was used in all experiments.

Sperm performance measurements

Sperm motility was analyzed using a Hobson Sperm Tracker (Hobson Tracking Systems Ltd, Sheffield, UK; e.g. see also Holt et al. 1997; Froman and Feltmann 2000; Kime et al. 2001). All observations were made with a microscope with a 4 x bright-field objective under pseudo dark-field conditions using a Ph3 annular phase ring, at a total magnification of 48.

First we used the Hobson Sperm Tracker to determine the concentration of motile sperm in the ejaculate. We diluted the sperm sample with Ringer-Lactate to a final concentration of $3*10^6$ motile cells/ml and kept it at 38 ˚C. Five minutes before each analysis of sperm swimming performance 100µl of this solution was diluted (10:1) with one of two blood-buffer suspensions that were previously prepared: one from a sister and one from an unrelated female, or both from unrelated females (see below). This final sperm dilution was kept at 38˚C until recording. Per female blood plasma sample, three separate sperm mixtures were prepared and sperm behavior was measured once in each of these three replicates.

Long-term sperm swimming performance is an important factor determining fertilization success in mallards (Denk et al. MS). Therefore sperm swimming was videotaped for 30 min after the sperm sample had been injected in a pre-heated (38 °C) MicroCell swimming chamber (50 µm depth; Conception Technologies, San Diego, USA), using a Sony SPT-M128 CE Black&White video camera mounted on the microscope and a Panasonic (NV-HS 900) Super VHS video recorder. Sperm was kept in the swimming chamber at 38 ˚C, slightly below avian body temperature (41 ˚C), to minimize effects of evaporation during the 30 min observation time. We measured the number of tracked sperm and straight-line velocity (VSL), which are both good predictors of paternity under sperm competition in mallards (Denk et al. MS), at eight

time intervals: 0-2 min, 4-6 min, 8-10 min, 12-14 min, 16-18 min, 20-22 min, 24-26 min and 28-30 min. The 'minimum track time' was set at 1.2 sec.

Experimental design

To examine the general effect of blood plasma on sperm swimming behavior we measured sperm performance (number of tracks and VSL) of eight ejaculates (one from each male) first in pure buffer solution and then in the buffer solution that included female blood plasma. For each test, blood plasma from a different female was used.

For further experiments, we used twelve pairs of females (N_{ducks}=24) such that one was a sister of an experimental drake and one was unrelated to the male. The blood of eight female pairs was also used to test sperm swimming performance of two to three different males, which were unrelated to both females. We obtained a total of 32 ejaculates from the eight males (on average 4 ± 1.3 SD per male, range: 1-5). Sperm was tested alternately in the blood plasma of the two females (sister or non-relative, respectively two non-relatives). Equal numbers from a given ejaculate were used in both tests. The order was randomly assigned for the first run (by tossing a coin) and then alternated for the replicates within the same ejaculate, as well as across the experiments.

For the above experiment blood was repeatedly collected from individual females and the reproductive status of the female (non-incubating / incubating) was recorded at each sampling date. Because this study took place during the reproductive season of mallards (April-May), we assumed that the non-incubating females were fertile (before or during egg-laying). Five ducks changed their reproductive status during the course of the study. In total, 30 blood samples were collected from 13 fertile ducks, and 34 samples came from 16 incubating females.

Statistical analyses

Repeatabilities (Lessells and Boag 1987) of the three replicate measurements of the number of tracks and VSL at every time interval varied between 0.48 and 0.79 (all $p<0.001$), suggesting that our methods were reliable and measurement error was low (see also Denk et al. MS). However, as previously observed (Denk et al. MS), the between-

ejaculate repeatabilities (same male, seasonal effect) are much lower (r=-0.04-0.32, 0.56>p>0.026).

To test whether there was a general influence of female blood plasma on sperm performance, we compared the number of tracks and VSL for sperm that was diluted in either pure buffer or in the blood-buffer solution using paired t-tests. To analyze the influence of male-female relatedness and female reproductive status on sperm performance we applied restricted maximum likelihood (REML) models with either number of tracks or VSL (averaged over the three replicates) as the dependent variable. As explanatory variables, we included the fixed effects relatedness (sister or unrelated), reproductive status (fertile or post-fertile), and order (whether a female was the first or the second to be tested). To control for multiple ejaculates per male we included male identity as a random factor in the model. Because of the varying ejaculate quality and because each ejaculate was used with a pair of females, we also included the term ejaculate identity in the random structure. Likewise, we added female identity as random factor, because the blood plasma of individual females was used with the sperm of up to four males. Each male-female pairing was unique and the interaction term with time interval was used to create a repeated measurement structure in the model. Repeating the analyses using the number of tracks and VSL averaged over the eight time intervals simplified the models, but did not change the conclusions (not shown). We initially constructed full models containing all explanatory variables. We then dropped non-significant terms from the model until the final model only contained variables with $p < 0.10$. Finally we re-added all eliminated terms to the final model to confirm their lack of significance. These p-values are reported here. To test whether there are consistent individual female effects on sperm performance, we compared the deviance generated by the final models with and without female identity in the random structure.

We used SPSS 11.5.0 (2002) for paired t-tests and Genstat 7.1.0.198 (Genstat 2003) for all other statistical analyses.

RESULTS

Effects of female blood plasma on sperm performance

A paired comparison of sperm performance diluted (10:1) in either pure TES-buffer or in the clear phase of the blood-buffer suspension clearly shows a stimulating effect of some component(s) of the blood plasma on sperm performance (Fig. 1). On average, as well as at each time interval, significantly more sperm were active in buffer with blood plasma than in the pure buffer (number of tracks averaged over the eight time intervals: paired $t_7=3.41$, $p=0.011$; each time interval separately: $t_7=2.94$ - 3.82, all $p<0.05$). In the absence of blood plasma, the number of tracks decreased by 68% within the first six minutes. In contrast, with blood plasma, the number of tracks decreased by 69 % over the entire observation period of 30 min. Overall, the number of tracks was about three times as high after adding blood plasma than in pure buffer. Similarly, adding female plasma had a positive effect on sperm swimming speed (VSL), increasing it about fivefold (Fig. 1; VSL averaged over the eight time intervals: paired $t_7=7.57$, $p<0.001$; each time interval separately: $t_7=6.30$ - 8.07, all $p<0.001$). In the presence of blood plasma, only a slight decrease in VSL (about 40%) was observed over the 30 min period, while in its absence a deceleration of 72% was observed already during the first six minutes.

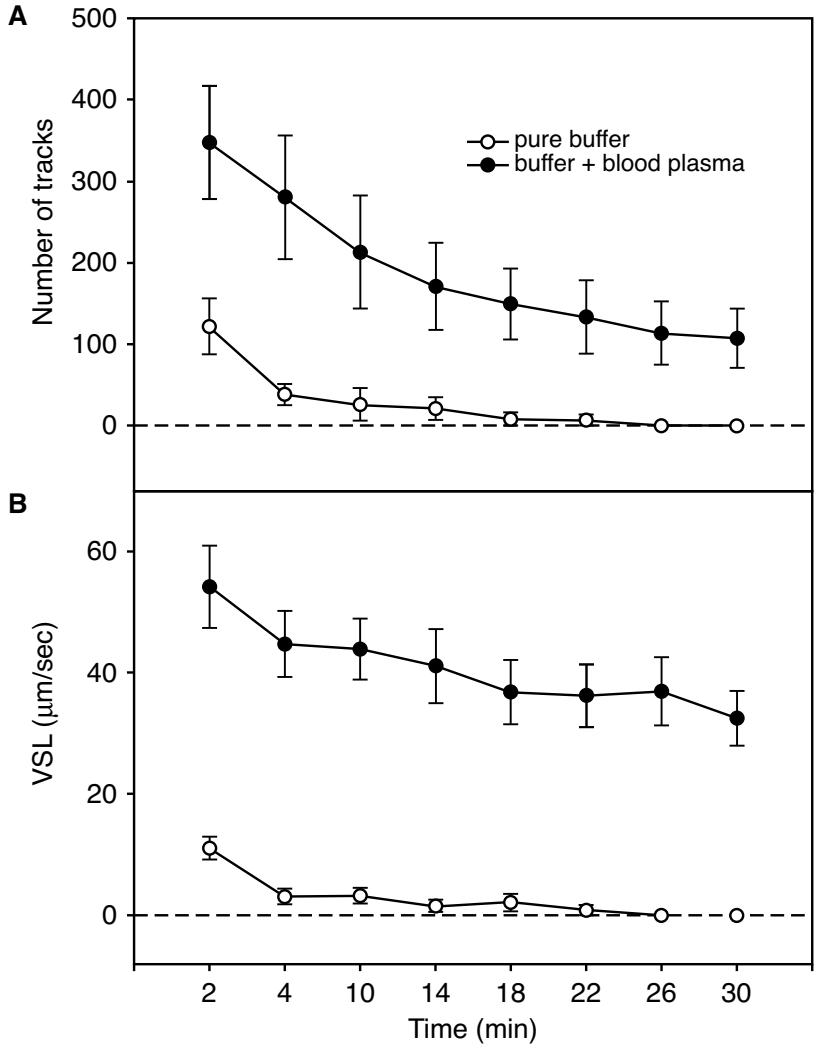

Figure 1. Average ± standard error of (A) number of tracks and (B) sperm swimming speed (VSL) of eight ejaculates from eight different males. Ejaculates were diluted either with pure buffer (open circles) or buffer plus blood plasma (filled circles).

Effects of relatedness and female reproductive status

Neither the number of active sperm cells (tracks), nor sperm swimming speed (VSL) was differentially affected by the blood plasma of a sister versus an unrelated female (Table 1, Figs 2 and 3). However, female reproductive status significantly influenced sperm swimming speed (Table 1, Figs 2 and 3). Sperm swam 14% faster in blood plasma from non-incubating (fertile) ducks than in plasma from incubating (post-fertile) females (Fig. 2). This effect was not attributable to an increase or decrease in the number of motile sperm, which did not differ between fertile and post-fertile ducks (Fig. 3).

	No. of tracks		VSL	
Independent factors	Wald	p	Wald	p
Relatedness	0.23	0.631	2.54	0.111
Reproductive status	0.14	0.707	23.44	<0.001
Order effect	23.49	<0.001	0.72	0.395
Random factors	Δ deviance	p	Δ deviance	p
Female	67.32	<0.001	34.06	<0.001
Male	2.23	0.14	0	1
Ejaculate	657.7	<0.001	255.3	<0.001
Male/Female x Time Interaction	568.54	<0.001	189.42	<0.001

Table 1. Influence of female blood plasma characteristics on sperm swimming performance. Results from REML-analyses with the number of motile sperm (tracks) and sperm swimming speed (VSL) as the dependent variables. For the fixed factors the Wald statistic is shown; for the random factors the change in deviance after excluding the factor from the final model is shown. Both are tested using a chi-square distribution with one degree of freedom. See Methods for further details.

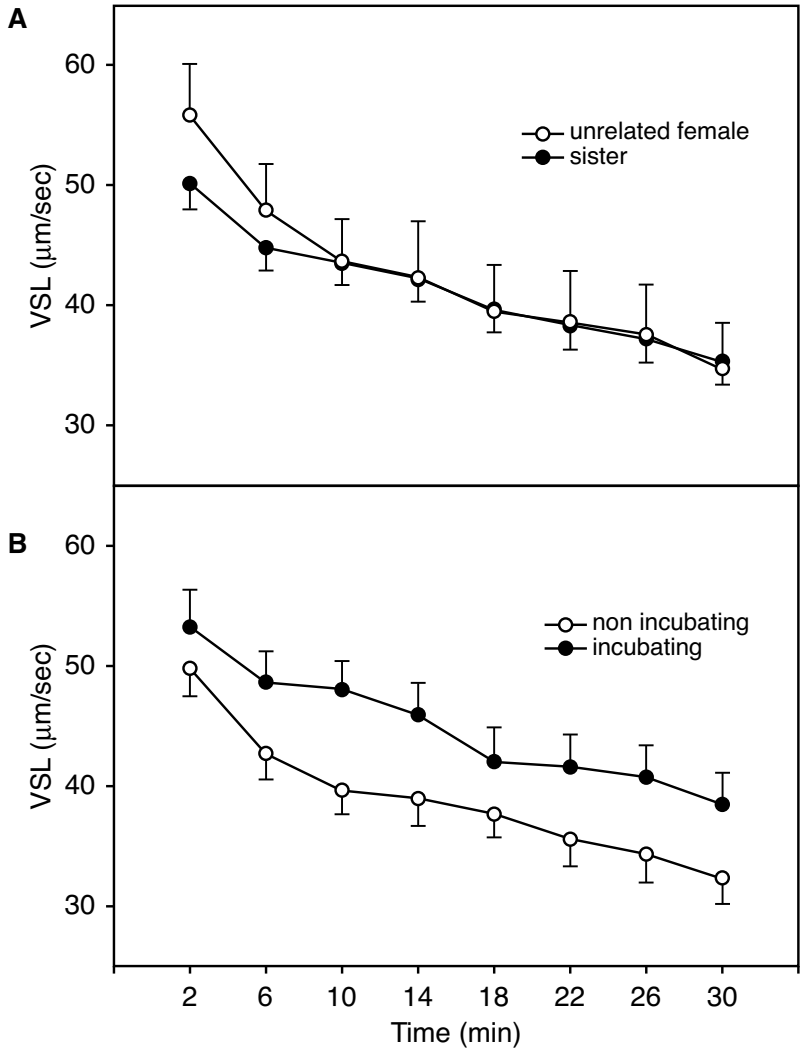

Figure 2. Effects of (A) relatedness (open circle: sister, filled circle: non-relative) and (B) female reproductive status (open circle: incubating, filled circle: fertile) on the average (± SE) sperm swimming speed (VSL) of 32 ejaculates (from eight different males) diluted in TES-buffer with female blood plasma.

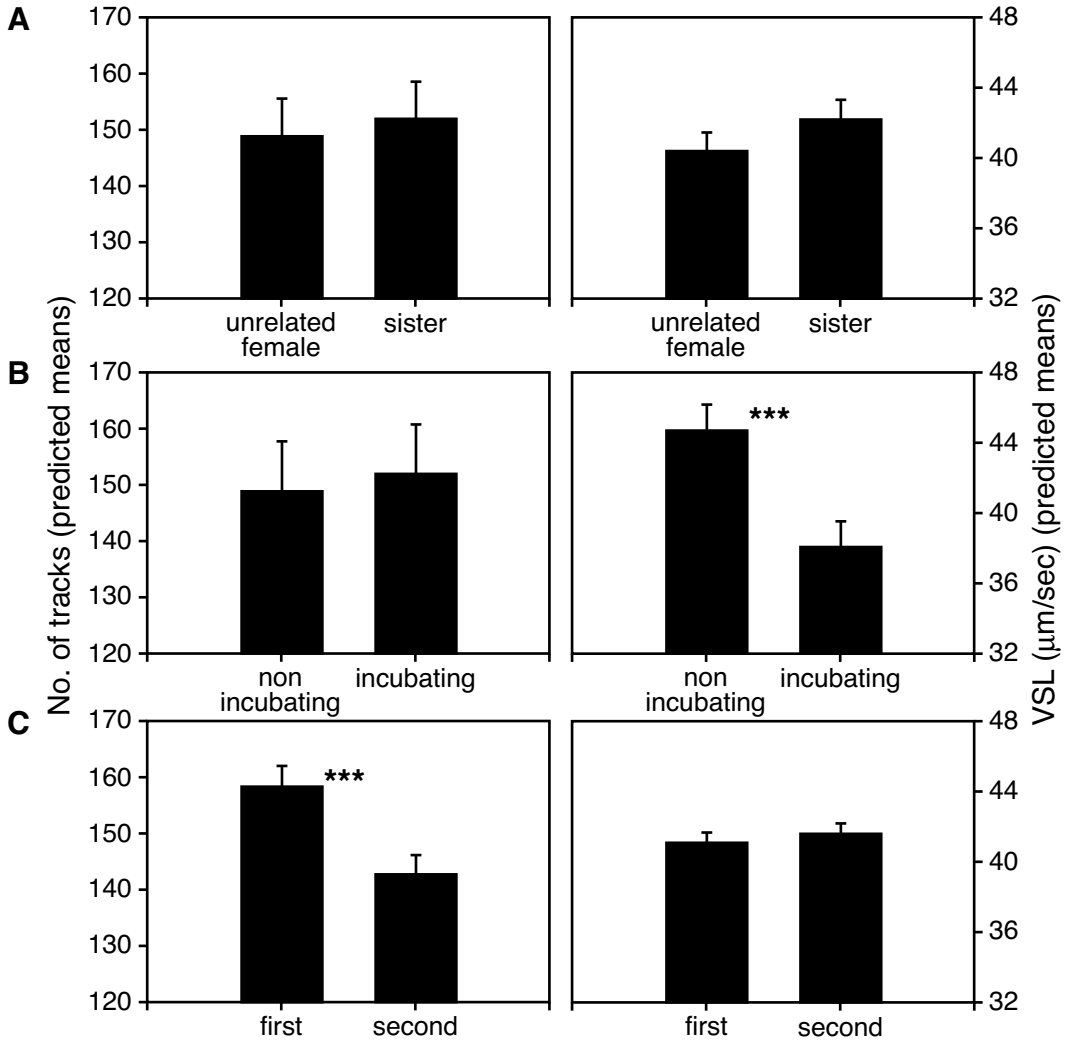

Figure 3. Factors influencing sperm performance (number of tracks and swimming speed, VSL). Shown are the predicted means +/- SE from the final models of the REML-analyses (see Methods for details). (A) Effect of relatedness. (B) Effect of the reproductive stage of the female. (C) Temporal order of measurement (averages over three replicates, 30 min difference between first and second). *** $p<0.001$.

Note that the number of tracks, but not sperm swimming speed, was affected by the order of the experiment: more sperm were active in the blood plasma of the first duck to be used in a test (Table 1, Fig. 3).

Effects of female identity

We found a strong general female effect on sperm activity while controlling for the fixed factors relatedness, reproductive status and experimental order (Table 1). This suggests that females exert an individual, differential stimulating influence on sperm performance.

DISCUSSION

This study examined the effects of individual female environments on sperm performance in a swimming chamber test. In general, female blood plasma stimulated sperm performance so that (1) a higher proportion of the sperm were active, (2) the sperm remained active over a longer period of time and (3) the sperm swam faster, compared to the performance in a standard buffer solution. Further experiments showed that this stimulating effect is (a) female-specific, (b) independent of the relatedness between the male (sperm) and the female and (c) stronger in fertile than in non-fertile females.

Our study thus shows that some unknown component(s) in the blood plasma can stimulate sperm and keep sperm cells active over a prolonged time period. Similarly, in humans supplementation with just blood serum increases sperm motility parameters (Delamirande and Gagnon 1991). However no effect of blood serum on sperm quality was detected in bovine (Verberckmoes et al. 2004) and canine sperm (Rijsselaere et al. 2004).

Focusing on birds, our results are similar to those of Froman and Feltmann (2000) on chicken in that the effect was not just transient, but rather resulted in a sustained sperm activity with only a slight decline over the 30 min time interval (15 min in case of Froman and Feltmann (2000)). However, the difference between our study and the one of Froman and Feltmann (2000) is that they used whole blood, including red blood cells. They ascribed the effect on sperm performance to the oxyhemoglobin of the erythrocytes,

which may facilitate mitochondrial respiration and therefore provide an energy source for sperm cells. Although we cannot exclude that such an oxygen-dependent process occurred in Froman and Feltmann's (2000) study, we clearly show that the blood plasma *per se* is sufficient to stimulate the sperm.

The two studies also differ in another way. We found differences in sperm activity levels already during the first two minutes after injection into the swimming chamber, whereas Froman and Feltmann (2000) observed identical sperm swimming speeds immediately after injection, followed by a dramatic decline in swimming speed in pure TES, but not in the buffer-blood suspension. This might be due to the different experimental protocols: Froman and Feltmann (2000) injected the sperm suspension immediately after dilution, whereas we diluted sperm five minutes prior to injection, which might have allowed the sperm to adapt to the physiological conditions of the buffer (with or without blood plasma). Comparing the behavior of rooster and mallard sperm relative to the time of dilution shows remarkably identical patterns: 5-7 min after dilution the VSL of rooster sperm was 18μm/sec in TES-only versus 50μm/sec in TES plus blood (Froman and Feltmann 2000), for mallard sperm the corresponding figures are 13μm/sec and 52μm/sec (this study). This suggests that the stimulating effect of the blood is due to a physiological effect caused by some substance(s) in the plasma, rather than an effect specific to the conditions in the sperm swimming chamber.

Our experiments show that the female physiological environment affects sperm performance. We further consider two issues. First, we discuss the advantages and relevance of using blood plasma instead of ovarian fluid. Obviously, sperm naturally interacts with the oviductal environment, not with the blood plasma. Secondly, we speculate which substances in the plasma might be responsible for the observed effects.

Although blood plasma imperfectly resembles the conditions that sperm experience in the female reproductive tract, many female-specific components are present both in blood plasma and in ovarian fluid. Studies in cattle show a tight relationship between concentrations of lactate, glucose, urea and ammonia in blood plasma and oviductal fluid (Kenny et al. 2002), and also similar concentrations of ions (calcium, magnesium, sodium) were found (Kenny et al. 2002). In other studies and species calcium, magnesium and potassium were reported in both fluids albeit in different

concentrations (see citations in Kenny et al. 2002). Moreover, transfer of steroid hormones (e.g. progesterone; Stefanczyk-Krzymowska et al. 2002), peptide hormones (e.g. oxytocin, relaxin, insulin), and prostaglandines between the reproductive and the blood system has been found in various mammalian species (Leese 1988; Menezo and Guerin 1997; Einer-Jensen and Hunter 2000 and citations therein). Also in birds a close anatomical linkage exists between the reproductive and blood system (e.g. Freedman and Sturkie 1963; Gilbert, Reynolds and Lorenz 1968). Calcium for eggshell formation is provided by the blood system and ovarian hormones (e.g. progesterone, estrogens) are present both in the oviduct and blood plasma (Whittow 2000). Although there might be differences in the concentration of these components, blood plasma represents an individual "fingerprint" with many substances also present in the ovarian fluid (Jeon et al. 2001). Therefore, we suggest that female blood plasma is a good substitute for ovarian fluid, at least for a first preliminary test of the influence of female physiology on sperm performance.

The use of blood plasma has several advantages. Firstly, blood samples can be taken easily and without any contamination. Particularly in ducks, it is difficult to collect samples of ovarian fluid without harming the animal and without pollution by contents of the cloaca. Secondly, blood plasma can be taken at any time, even during egg laying. Thirdly, blood can be collected repeatedly from the same individual. Earlier studies in domestic hens, which used fluid of the ovarian pockets, had to sacrifice the animals to collect the fluid (Ashizawa and Wishart 1992).

We observed that the stimulating effect of blood plasma on sperm swimming speed was higher when the plasma came from a fertile duck (before or during egg-laying) than from a post-fertile, incubating duck. During egg-laying circulating calcium-levels peak in birds in general (Whittow 2000). In mallards, serum calcium content during egg laying (0.219 mg/ml) is twice as high as during incubation (0.103 mg/ml; Fairbrother et al. 1990). Because calcium is one of the major sperm activating factors in birds (Ashizawa et al. 2000), the elevated calcium concentration could potentially cause the observed effect. However, this is unlikely in our experiment, because the blood plasma is 100-fold diluted in Ringer-Lactate buffer, which in itself contains high levels of calcium (0.072 mg/ml). Thus, variation in calcium content in the blood plasma is only going to

have a marginal effect on the total calcium concentration in the buffer solution. The same is true for the other ions like sodium.

We suggest that female hormones might be responsible for the stimulating effect of sperm performance. Female hormones are not present in the buffer solution, but were added by the dilution with female blood. Changes in the hormonal constitution of female birds, in particular ducks, before and during incubation are well documented (Whittow 2000). In mallards, prolactin levels increase, whereas progesterone drops dramatically with the onset of incubation (Hall 1991). Although to our knowledge no effect of female hormones on sperm activity in birds has been documented, a sperm stimulating quality of progesterone has been reported for mammalian sperm (e.g. Jaiswal et al. 1999, but see Jeon et al. 2001; Wang et al. 2001). Further experimental investigations are needed to validate the potential sperm activating influence of hormones, particularly those that are elevated during the fertile period of females.

The relatedness between the female and the male did not bias sperm performance. Therefore, differential sperm activation based on genetic similarity seems less likely as a mechanism of cryptic female sperm selection in mallards, at least via substances that are also found in the blood plasma. This corroborates earlier experimental studies, which failed to show female sperm selection based on sperm genotype in this species (Cunningham and Cheng 1999; Denk et al. MS).

Even though female blood plasma did not provide a discriminating environment for sperm selection based on relatedness, there were general differences between females in their sperm activating properties. Further detailed research has to be conducted to a) validate the use of blood plasma as a substitute of oviduct fluid and b) to identify factors in the female blood plasma, which are responsible for differential sperm stimulation (e.g. hormones, serum proteins, glucose or ATP content).

Although we are aware of the limitations of our study, our results show that component(s) present in the female blood plasma have properties that mediate sperm performance and differ between females. Sperm motility may not be an absolute male trait, but rather be shaped by the interplay with the female environment. Further experimental studies and the application of a more adequate substrate (ovarian fluid) are

needed to validate our findings and to further investigate mechanisms and consequences of female influence on sperm performance.

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CHAPTER FOUR

EJACULATE FREQUENCY AFFECTS SPERM QUANTITY AND QUALITY IN MALLARDS

Angelika G. Denk, Alois Holzmann, Etiënne L. M. Vermeirssen & Bart Kempenaers

ABSTRACT

Behavioral observations and genetic evidence reveal that in many bird species females copulate with multiple partners. In such cases, male competition over fertilization continues even after copulation. The outcome of sperm competition depends both on the number of transferred sperm and on sperm quality. However, males are not unlimited in their ability to deliver optimal ejaculates and trade-offs exist between ejaculate frequency and quality. Here we report that sexual abstinence significantly increases sperm concentration and swimming speed in ejaculates of wild mallards (*Anas platyrhynchos* L.). Increased competitiveness of ejaculates due to infrequent copulations may be particularly beneficial for unpaired mallard drakes, which are commonly observed in male-biased populations of this socially monogamous duck species. These males are known to engage in forced extra-pair copulations and our study suggests that they may have a competitive advantage over paired males.

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INTRODUCTION

When a female copulates with several males, sperm from these males will compete within the female's reproductive tract to fertilize her ova (Parker 1970). If sperm competition is a raffle the number of transferred sperm will influence a male's fertilization success (Parker 1990). Indeed, there is evidence that sperm production is greater in species where sperm competition is more intense (Birkhead and Møller 1992, Parker et al. 1997) and individuals adjust the amount of transferred sperm according to the risk of sperm competition (Parker et al. 1997, Evans et al. 2003, Pizzari et al. 2003). However, not only sheer numbers, but also sperm quality determines fertilization success: males with more mobile or faster swimming sperm obtain more paternity than their opponents in direct competition with equal sperm numbers (domestic fowl *Gallus gallus* (Birkhead et al. 1999); turkey (Donoghue et al. 1999); mallard *Anas platyrhynchos* (Denk et al. MS).

The production of ejaculates costs time and energy and males cannot produce limitless numbers of sperm (e.g. Dewsbury 1982; review in Wedell et al. 2002). Thus, the frequency of ejaculation can have a nontrivial impact on sperm number and quality and hence on the competitive ability of a male's ejaculate. Some studies on mammals have found negative associations between copulation rate and sperm numbers per ejaculate (e.g. Bencheikh 1995; Preston et al. 2001; Nizza et al. 2003) or sperm quality (motility and swimming speed: Bencheikh 1995; Thwaites 1995; Ambriz et al. 2002).

In birds, few studies have investigated the relationship between copulation frequency and sperm quality, and the results are mixed. In zebra finches (*Taeniopygia guttata*), Birkhead et al. (1995) found that sperm motility and swimming speed were negatively influenced by ejaculation frequency. Contrary, in domestic chicken, individuals with a frequent ejaculation treatment had only marginally reduced sperm concentrations, and their sperm motility was higher than in birds with low ejaculation frequency (Fan et al. 2004). However this effect was only observed during the first two weeks of a four-week experiment and no significant difference was found during the last two weeks (Fan et al. 2004).

Although in general, frequent copulations seem to reduce sperm concentration and sperm quality in a single ejaculate, the total amount of sperm inseminated via

frequent copulations may still be higher (weekly sperm production: Bencheikh 1995; Fan et al. 2004). Therefore, two different male sperm allocation strategies are conceivable. (1) Males may pursue frequent copulations with an overall high sperm output at the expenditure of the quality and competitive ability of a single ejaculate. This strategy might be typical for paired males. (2) Males may focus on rare copulation events, but at these single occasions they might be disproportionately successful due to superior ejaculates. This strategy may be used by unpaired males or by paired males that perform extra-pair copulations outside the fertile period of their social mate.

Mallards are a socially monogamous species, but wild populations are typically male-biased and several drakes remain unpaired (Bellrose et al. 1961; Denk and Kempenaers MS). Extra-pair copulations are the only possibility for these males to reproduce. Unpaired and paired males are equally likely to attempt forced extra-pair copulations (captive mallards: Davis (2002); wild population: Cunningham (2003)) and the percentage of nests containing extra-pair young is high (an estimated 48-56%: Evarts and Williams (1987); Denk and Kempenaers MS). If copulation frequency negatively influences ejaculate quality, unpaired males may have a competitive advantage over paired males in gaining extra-pair paternity.

We investigated the impact of ejaculation frequency on sperm competitive ability, by examining ejaculates of individual mallard drakes when their social partner was present (opportunity to copulate at a natural frequency) and after removal of their mate (only one ejaculate per week). We here report how ejaculate characteristics changed with treatment.

MATERIAL AND METHODS

The drakes used in this experiment (N=5 yearlings and N=4 two-year olds) were direct descendants of free-living mallards, but were hatched and raised in captivity (see Denk et. al MS). Before the experiment (August 2001 – January 2002), birds were kept in large outdoor aviaries (120-165 m^2) in mixed flocks of equal sex ratios (24-34 animals), and allowed free mate-choice during autumn. From the end of January onwards, nine unrelated males were isolated with their female partner in small aviaries (2.4-6.1 m^2,

including a water tub). From 24 April 2002 onwards, males were kept singly in the same aviary.

The animals were not disturbed, except for feeding and cleaning of the aviaries, and for sperm collection (see below). During the breeding season ducks were fed commercial duck food particularly aligned for nutritious needs during reproduction (Anseres 4, Kasper Faunafood, Woerden, Netherlands) mixed with wheat. At least three times a week ducks received fresh lettuce and fresh mealworms were given as a reward after sperm collection.

From February until mid of April drakes were trained for sperm collection to familiarize them with regular capture and handling for artificial sperm collection. We obtained sperm by following the massage procedure described in Lake and Stewart (1978). We collected sperm in a 1ml syringe at the base of the intromittent organ, where the sperm is ejaculated. To minimize fecal contamination of ejaculates we removed food the evening before the day of sperm collection. Food was replaced immediately after sperm collection and served as additional reward.

Sperm was collected for analyses (a) in two consecutive weeks in April when the social partner was still present and (b) after female removal on a weekly basis until early June. During the first phase of sperm collection, females were removed one day before sperm collection to prevent copulation shortly before artificial stimulation, and returned immediately after sperm collection. Because we used the ejaculates for artificial inseminations, we considered only ejaculates containing at least 30% motile sperm (N=56 ejaculates) for further analysis. In 22 cases we failed to obtain an ejaculate or sperm were immotile. This was independent of the presence of the drake's social partner (7 out of 20 sperm collections (35%) failed when the female was present, whereas 15 out of 58 (26%) failed in her absence; Fisher's exact test: $p=0.59$). Sperm number and quality was determined as described in Denk et al. (MS). Briefly, sperm concentration was determined by counting three replicates of two sperm dilutions in an improved Neubauer counting chamber (repeatability $r=0.93$, $n_0=6$, $p<0.001$, N=56 ejaculates). Sperm quality was analyzed using a computer-assisted sperm motion analysis system (Hobson Sperm Tracker; Hobson Tracking Systems Ltd, Sheffield, UK). As sperm diluent we used Ringer-Lactate solution (Ringer-Lactat after Hartmann, Z.Nr. 1-19.566, Mayrhofer

Pharmazeutika GmbH, 4020 Linz, Austria) (Humphrey 1972). For measurement of sperm quality, semen was diluted 10:1 with a TES-buffered saline solution containing blood plasma of an unrelated female to a final concentration of 3 million cells per ml. Sperm swimming in a MicroCell swimming chamber (50 µm depth; Conception Technologies, San Diego, USA) was videotaped for 15 min and in 2 min intervals straight line velocity (VSL) was measured (measurement repeatability r=0.74-0.85, n_0=3, N=56 ejaculates, all $p<0.001$).

To test the effects of isolation from the social partner on ejaculate characteristics (sperm concentration, sperm swimming speed), we used mixed models with male identity as a random factor. To test seasonal effects, date was included as a covariate. For sperm swimming speed each of four time intervals (0-2min, 4-6min, 8-10min, 12-14min) was analyzed separately. Male age was included in the initial models, but did not explain variation in sperm numbers or quality; age is therefore not mentioned further. We used Genstat 7.1.0.198. (Genstat 2003) for all statistical analyses.

RESULTS

Both sperm concentration and swimming speed were significantly higher when males were kept isolated from their social partner and natural copulations were prevented (Figs 1 and 2, Table 1). When males delivered only one ejaculate per week after artificial stimulation, sperm concentrations were more than twice as high as in ejaculates obtained by the same method when natural copulations were possible during the week before sperm collection (Fig. 1). Straight-line velocity of swimming sperm also increased significantly after natural copulations were prevented (Fig. 2; Table 1). No seasonal trend in sperm concentration and swimming speed was observed (Table 1); only in ejaculates of the isolated males a seasonal decline in long-term swimming speed (VSL 12-14 min) was observed (N=42 ejaculates; REML: Wald F_1=5.7, p=0.02).

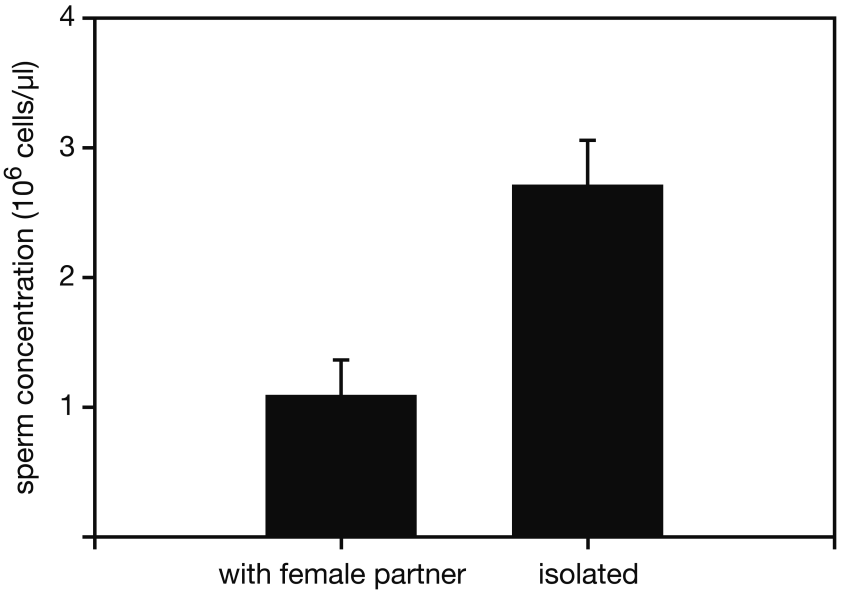

Figure 1. Removal of the social partner increases sperm concentration in mallard ejaculates (paired *t*-test, N=9 males, t=2.8, p=0.02). Shown are mean and standard error.

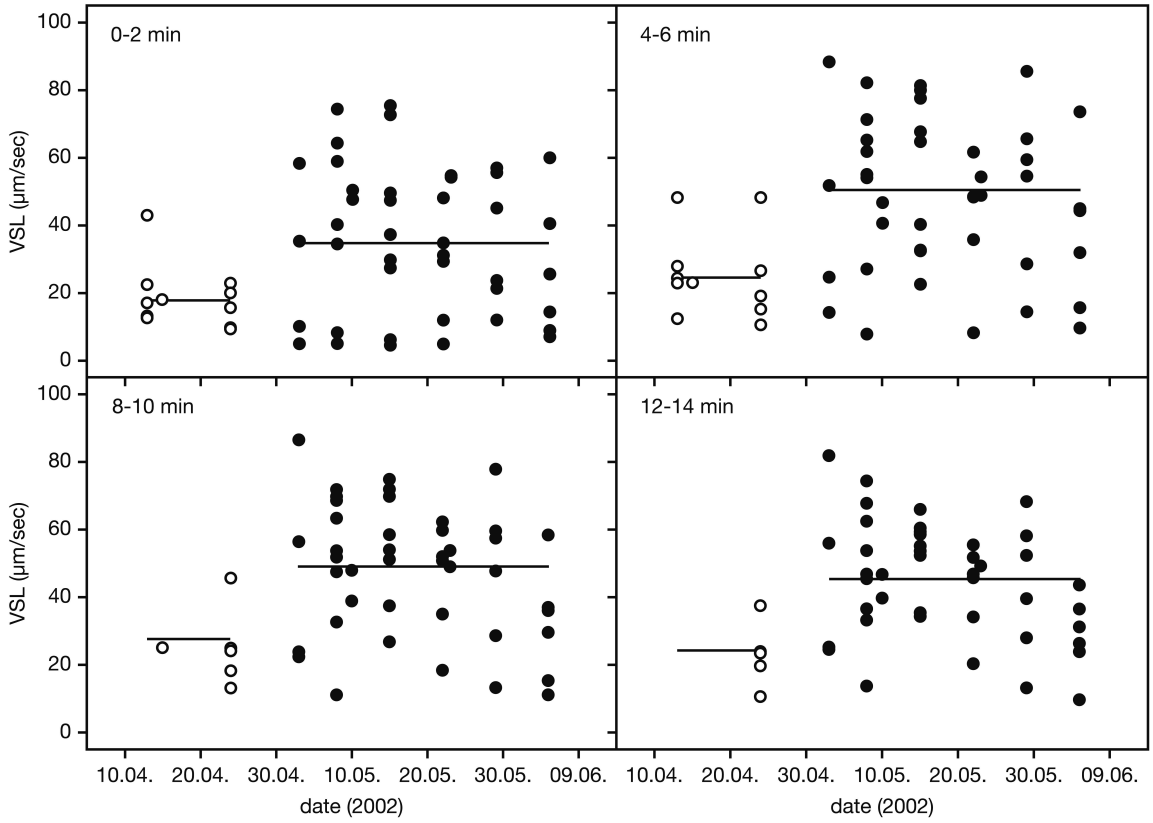

Figure 2. Removal of the social partner increases sperm swimming speed (VSL) at all four observation intervals. Unfilled symbols indicate “male plus female partner”; filled symbols indicate “male isolated”. Means predicted by the REML model (solid lines) are shown for illustration. For statistical details, see Table 1.

	Female effect		Seasonal effect	
Ejaculate characteristic	Wald F	p	Wald F	p
Sperm concentration	13.0	<0.001	0.1	0.79
Sperm swimming speed (VSL)				
VSL 0-2 min	7.9	0.005	1.2	0.31
VSL 4-6 min	19.6	<0.001	0.9	0.49
VSL 8-10 min	9.9	0.002	1.2	0.28
VSL 12-14 min	8.4	0.004	1.5	0.15

Table 1. Influence of the presence of the social mate on ejaculate characteristics in nine mallards (N=56 ejaculates). The Wald statistic is shown, which follows a chi-square distribution with df=1.

DISCUSSION

Ejaculate quality, measured as sperm concentration and sperm swimming speed, increased in mallard drakes after we removed their social mate. Both traits, which are known to be important determinants of success in sperm competition, thus depended on the copulation behavior of the male rather than being a constant male attribute.

Although we sporadically observed copulations in the aviaries, we did not collect data on copulation frequencies for all birds. We nevertheless assume that the changes in ejaculate quality were a direct consequence of a change in copulation frequency after female removal. However, we cannot exclude that the changes are caused by a male's "decision" to invest more in sperm production after the "loss" of his social mate. This seems unlikely, because spermatogenesis itself takes too much time (e.g. 12.8 days in Japanese quail *Coturnix coturnix japonica* (Lin and Jones 1992)). Therefore, changes in

sperm concentration resulting from a decision to upregulate spermatogenesis would not be detectable within nine days as observed in our study.

One could also argue that removing a duck from the aviary might have lowered social stress and resulted in increased sperm quality. However, this seems unlikely because males were kept together with their freely chosen social mate and neither aggressive behavior nor other signs of social stress were detected (e.g. missing feathers). Similarly, increased food availability appears unlikely to have caused elevated ejaculate quality, because ducks were always provided food ad libitum and malnutrition seems to have no effect on semen quality, at least in mammals (Thwaites 1995).

Ejaculation frequency can limit the amount and quality of transferred sperm due to short-term effects like depletion of the male's sperm store (Birkhead 1991). This was observed in zebra finches, following several ejaculations in rapid succession (Birkhead et al. 1995). Birkhead et al. (1995) also reported a gradation of sperm quality within the seminal glomera, such that sperm motility increased towards the region nearest to the cloaca. This gradient was attributed to a maturation and sorting process.

Lack of maturation time might also account for the long-term decline in sperm motility after an extended period of frequent copulations (see also Ambriz et al. (2002)). In birds sperm are still immotile, when they are released from the seminiferous epithelium (Ashizawa and Sano 1990). Maturation of avian sperm is envisioned to occur at the passage of the efferent ducts, where also sperm concentration increases due to absorption of the seminiferous tubule fluid (Whittow 2000). If ejaculations occur at a high frequency, this passage and residence time within the drake's sperm store in the ducti deferentes might be reduced. Therefore sperm release may take place before sperm maturation and concentration are fully completed.

Limitations on sperm production can have severe effects on male reproductive success in wild populations. Preston et al. (2001) showed that copulation rates in a wild population of male soay sheep (*Ovis aries*) are negatively associated with sperm numbers per ejaculate. Thus, during sperm competition sperm of dominant, frequently copulating males can be outnumbered by sperm transferred in ejaculates of behaviorally subordinate males with intact sperm reserves (Preston et al. 2001). While copulation frequencies of

dominant males increase during the reproductive season, male limitation on ejaculate production accounts for the decline in the proportion of lambs sired by these males.

Similarly the success of paired and unpaired mallard drakes may be influenced by copulation frequency. By observing a wild population of mallards, Cunningham (1997) estimated that pair copulations occur at a rate of 1.38 per day, whereas forced extra-pair copulations are half as frequent (0.78 per day). If these extra-pair copulations are pursued by paired and unpaired males at the same rate (Cunningham 2003), it can be assumed that on average paired males copulate more than four times as frequent than unpaired males. Because unpaired males copulate at a lower frequency they may deliver ejaculates of superior competitiveness compared with frequently copulating males and might be disproportionately successful in sperm competition at these rare occasions. Further observational and genetic analyses of paternity in wild mallard populations will be necessary to evaluate this hypothesis.

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CHAPTER FIVE

TESTOSTERONE AND TESTES SIZE IN MALLARDS (*ANAS PLATYRHYNCHOS* L.)

Angelika G. Denk & Bart Kempenaers

ABSTRACT

The steroid hormone testosterone mediates the expression of many secondary sexual characters including behaviors, which influence male reproductive success. Testes are one of the major sources of androgens, in particular of testosterone. Although a positive relationship between testes size and testosterone levels could be hypothesized, it has rarely been tested intraspecifically. We investigated this link in mallards using a double-antibody radio-immuno-assay (RIA) to measure hormone levels and x-rays to determine testes size. Here we report a positive correlation between both traits in a group of 13 drakes during the reproductive season. Although the evolutionary causalities leading to this correlation are unclear, this relationship may have important implications for female mate choice. Female mallards prefer to mate with males with high spring testosterone levels. These males may better guard the female against forced extra-pair copulations, but females may also indirectly select for males with larger testes and hence a greater ability of sperm production.

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INTRODUCTION

The testes fulfill two important functions within the male body: spermatogenesis takes place in the seminiferous tubules of the testes (Whittow 2000) and the production of androgens (e.g. secretion of testosterone (T)) occurs in the Leydig cells of the interstitial tissue (Lofts and Murton 1973). Both gonadal functions strongly affect male reproductive success through male-male competition and mate choice.

Sperm production and hence number of sperm transferred to the female is an important factor affecting fertilization success in situations of sperm competition (Parker 1990). If testes size reflects sperm producing ability, as for example shown in domestic fowl (*Gallus domesticus*; de Reviers and Williams 1984), one can expect that gonadal size increases with increasing intensity of sperm competition. Indeed, interspecific comparative studies in various taxa support this prediction (e.g. birds: Møller 1991; specifically waterfowl: Coker et al. 2002; primates: Harcourt et al. 1981, Harcourt et al. 1995; frogs: Byrne et al. 2002; fish: Stockley et al. 1997; butterflies: Gage 1994). Intraspecific variation in testes size also positively correlates with sperm competition intensity (Hosken and Ward 2001; Brown and Brown 2003) and success in obtaining paternity (Preston et al. 2003; Schulte-Hostedde and Millar 2004).

Production of the sex steroid T influences male mating success (Andersson 1994) since T regulates the expression of many secondary sexual characters and sexual behavior (Wingfield et al. 2001). Because of immune-suppressive properties of T, traits influenced by this sex steroid may honestly reflect male quality (superior immune system), an idea formulated in the immunocompetence handicap hypothesis (Folstad and Karter 1992). Although the general validity of this concept requires further (experimental) studies (Roberts et al. 2004), it is clear that some sexually selected signals are T-dependent (e.g. Peters et al. 2000; Gonzalez et al. 2001).

An interspecific analysis across 116 bird species revealed a positive association between T levels and testes size (Garamszegi et al. 2004), but only little information exists on the intraspecific level. In free-living red-winged blackbirds, testes size did not correlate with levels of circulating T, nor was there evidence for a relationship between testes size and parasite infection (Weatherhead et al. 1993). Some studies indirectly linked testes size with T levels taking size of secondary sexual traits and levels of parasite

infection as indirect measures of circulating sex steroids. In greenfinches (*Carduelis chloris*), males with larger testes had a brighter yellow plumage and were more likely to be infected by haematozoan parasites (Merila and Sheldon 1999). Although variation in plumage brightness is not necessarily caused by variation in T levels, both variables may be correlated and the results of Merila and Sheldon (1999) could be interpreted as males with larger testes signaling phenotypic quality at the cost of reduced immunocompetence caused by high T levels. However, in roosters a negative relationship between testes size and the intensity of helminth infections was observed (Zuk et al. 1990), leaving conclusions of links between testes size and T levels ambiguous.

The aim of this study was to investigate whether an intraspecific correlation between testes size and circulating T levels exists. As a model species we chose mallards, a socially monogamous duck species that exhibits frequent extra-pair copulations (McKinney et al. 1983; McKinney and Evarts 1998). Levels of extra-pair fertilization are high (Evarts and Williams 1987; Denk and Kempenaers MS) and selection should thus favor high levels of sperm production. In mallards females show strong preferences for males with higher T levels (Klint 1985; Schmedemann and Haase 1985; Klint et al. 1989; Davis 2002b). A tradeoff between T-dependent male signals and immune investment may exist in this species, because a nonpathogenic immune challenge caused a substantial decline in T levels (Peters et al. 2004a). Therefore, if high levels of T constitute a high cost in this species, testes size, which can be selected to maximize sperm production, and T secretion might be decoupled. Alternatively, if both traits are linked, T-dependent traits may not only represent an honest signal of immune competence, but also indicate high levels of sperm production.

MATERIAL AND METHODS

Drakes were direct descendants of five pairs of wild mallards (caught at Lake Starnberg (47º54’N, 11º18’E) and Lake Ammer (48º00’N, 11º08’E), Southern Germany). All individuals used in this study were yearlings, which we had hatched and raised in captivity (see Denk et. al MS). Kinship of the drakes was known and confirmed by

microsatellite analysis (see Denk et al. MS). We used on average 2.6 males per family (range: 1–5).

Until the beginning of the breeding season (March 2001) we kept mallards in two mixed-sex flocks to allow normal sexual maturation (aviary size: 120 m^2 and 405 m^2). At the start of the reproductive season, we separated males and females in adjacent aviaries, so that visual and acoustic contact remained possible. We kept the males in two groups: 30 individuals in an aviary of 150 m^2 and 15 individuals in an aviary of 52 m^2. Ducks were habituated to human presence in the aviaries and to regular capture. We provided ad libitum commercial duck food particularly designed for nutritious needs during reproduction (Anseres 4, Kasper Faunafood, Woerden, Netherlands) mixed with wheat. At least three times a week ducks received fresh lettuce.

We collected blood samples from nine males in the first group (7 May 2001), and from four males in the second group (8 May 2001). Blood was taken between 12:30 and 15:30, when plasma T levels show their daily peak during the reproductive season in mallards (Balthazart and Hendrick 1979). Immediately after blood collection we centrifuged blood samples for 3 min at 2000 g. After transfer to the lab, we centrifuged the samples for another 5 min at 13000 g before we separated blood plasma from blood cells and stored it at –70˚C until analysis. VetMedLabor (Ludwigsburg, Germany) determined T concentration using a direct double-antibody radioimmuno-assay (RIA; DSL-4100, Diagnostic Systems Laboratories; for details see Peters et al. 2004a).

After blood collection we measured beak length (from tip of the beak to the start of the feathers) and width, head length and tarsus length using calipers, and foot size using a ruler. We took all measurements to the nearest millimeter and weighed all birds to the nearest gram (balance: Sartorius AG Göttingen).

One-two days after blood collection (8 and 10 May) a veterinary (Dr. Fries, Pöcking, Germany) x-rayed (62 kV, 0.05 sec) the drakes from the dorsal side. We measured testis length and width on the x-ray images using calipers (left and right testis; to the nearest 0.1 mm). We calculated testis volume using the equation

$$V = 4/3 * \pi * a^2 * b$$

where a is length and b is width (assuming an ellipsoid testis shape; see Møller 1991). We used the mean specific gravity of bird testes (1.087 g cm^{-3}; Møller 1991) to estimate testis mass.

To analyze the relation between testes size (testes mass) and levels of circulating T, we used mixed models (REML). There was some indication of a family effect on T-level (oneway ANOVA: $F_{4,8}$=4.93, p=0.027). Therefore we controlled for family effects by including family identity as a random factor in the model, even though the change in deviance after including/excluding it from the model was not significant (p=0.15). We also included aviary as a random factor; this effect was not significant (p=0.86) and we excluded it from the final model.

To control for allometric effects of testes size we used "relative testes mass" calculated as total testes mass (sum of right and left testis) divided by body mass. We repeated the analysis with absolute total testes mass as the dependent variable, and body mass as a covariate. Both methods yielded similar results, so we only report the latter. The relationships between total testes mass and body condition respectively structural size were analyzed in the same way. As a measure of structural size we used the first principal component (PC1) explaining 83.6 % of variation in bill length and width, head, tarsus and foot length. For REMLs and principal component analysis we used Genstat 7.1.0.198 (Genstat 2003); we performed all other statistical analyses with SPSS (12.0.1). All tests are two-tailed.

RESULTS

Testes exhibited significant asymmetry, with the left testis being larger than the right one in all 13 individuals (Table 1). Size of left and right testis was positively correlated (Table 1). Testes mass was not associated with body mass (REML: Wald F_1=0.02, p=0.89) or structural size (REML: Wald F_1=0.01, p=0.92). Plasma levels of T were positively correlated with total testes mass (REML: Wald F_1=5.17, p=0.023; Figure 1), while controlled for body size (REML: Wald F_1=8.67, p=0.003).

Testis	Left	Right	paired t-test	Pearson correlation
length [cm]	5.0±7.4	4.3±8.5	t=7.52, p<0.001	r=0.93, p<0.001
width [cm]	2.4±0.3	2.3±0.3	t=2.52, p=0.027	r=0.91, p<0.001
volume [cm^3]	15.5±6.7	12.4±6.3	t=5.96, p<0.001	r=0.91, p<0.001
mass [gram]	16.6±7.2	13.3±6.8	t=6.02, p<0.001	r=0.96, p<0.001

Table 1. Measurements of left and right testes in 13 mallard drakes during the reproductive season.

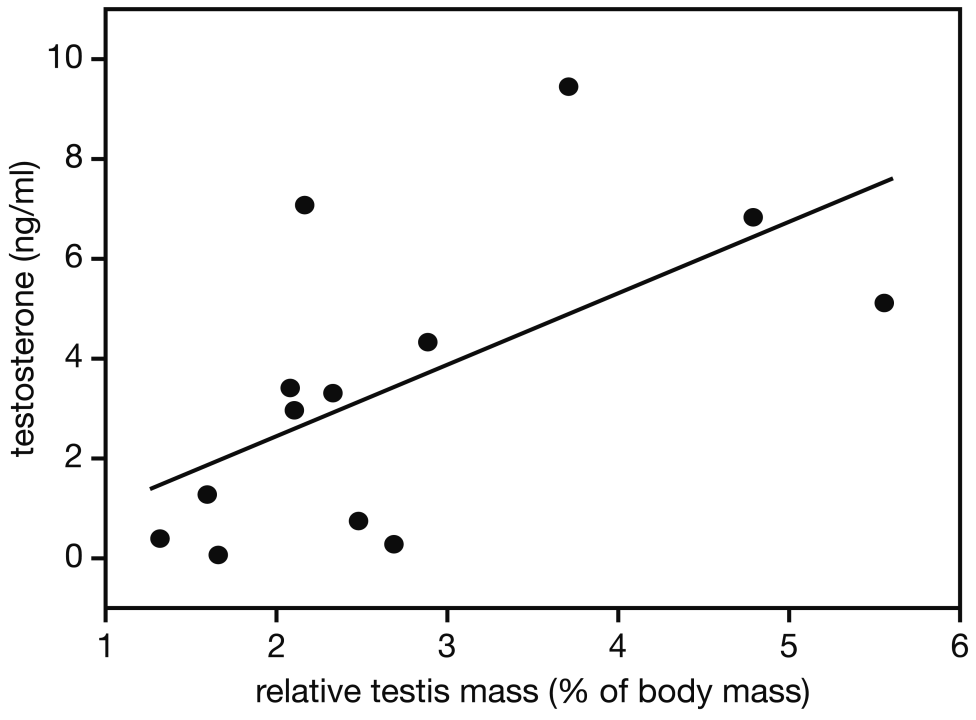

Figure 1. Levels of testosterone in blood plasma increase with relative testis mass.

DISCUSSION

This study shows a positive correlation between relative testes size and plasma levels of circulating T in a group of male mallards during the reproductive season. A similar positive relationship was shown in a comparative study across 116 bird species (Garamszegi et al. 2004). Species with smaller relative testes size have lower peak plasma T levels compared with species with larger relative testes size. Here we show that this relationship also exists at the intraspecific level. However the causality remains unclear. High T levels may regulate testes size, as they influence the secretion of gonadotropin-releasing hormones (Wingfield and Moore 1987) and are found to prevent testicular regression (Lofts and Murton 1973). Alternatively larger testes may simply contain more hormone producing tissue (Leydig cells) and therefore produce more androgens.

Garamszegi et al.'s (2004) comparative analysis suggested that peak testosterone level, level of extra-pair paternity and relative testes mass coevolved. They hypothesized that the most likely evolutionary scenario is that testes mass primarily evolved to allow intense sperm competition and increased T production was secondly favored for defending females against threats of extra-pair copulations. Although our study does not allow to evaluate this, we discuss potential implications of a correlation between testes size and T levels in mallards.

Mallards show high levels of extra-pair paternity (Evarts and Williams 1987; Denk and Kempenaers MS), suggesting that sperm competition is intense. Success in sperm competition is partly determined by the numbers of inseminated sperm (Parker 1990). Maximizing sperm numbers can be achieved by increasing the testes size, but also variation in T-levels may influence sperm numbers, because T in synergy with FSH (follicle-stimulating hormone) promotes sperm production (Wingfield and Moore 1987; McLachlan et al. 1996; Kirby and Froman 2000).

Fertilization success under sperm competition also depends on sperm quality (in mallards, see Denk et al. MS), which may also be influenced by T levels. This can happen in two ways. First, high T levels may down-regulate the immune system (Folstad and Karter 1992), thereby reducing autoimmune reactions against own sperm (Folstad and Skarstein 1997; Hillgarth et al. 1997). Second, T reduces the immune defense against

parasites (Folstad and Karter 1992, but also see Roberts et al. 2004). This can be crucial under sperm competition, because even infections with non-gonadal parasites negatively affect ejaculate quality (e.g. nematode infection in arctic charr (*Salvelinus alpinus*): Liljedal et al. 1999; further citations see Folstad and Skarstein 1997). Therefore only males with high parasite resistance may be able to afford high levels of circulating T without reducing sperm quality and hence ejaculate competitiveness.

High levels of extra-pair paternity also mean challenges for males at the behavioral level. Males have to guard and defend their social mate, and might also increase their fitness by pursuing extra-pair copulations themselves. Both behaviors are T dependent. In mallards, males with high levels of T are better at defending their social mate (Davis 2002a), and forced copulation behavior is positively correlated with T levels (Davis 2002b). During pair formation in autumn (Williams 1983), females prefer drakes that have high T levels in spring (Davis 2002a), probably because they benefit through increased protection against forced copulation attempts by other males (Davis 2002a). However, assuming a positive correlation between T levels and testes size, females may also benefit because their males will have a higher sperm producing ability. This may be advantageous for females in two ways: first they may ensure fertilization by their mate (even after forced copulations) and reduce the risk of sperm depletion. Second, if testes size is a paternally inherited trait, females would produce sons that have a reproductive advantage. Also other cues (yellow bill), which indicate the male's future reproductive quality (sperm quality) (Peters et al. 2004b), were found to be important for mate choice in autumn (Omland 1996a, 1996b).

In waterfowl species, a positive correlation between the occurrence of extra-pair paternity and testes size has been reported (Coker et al. 2002). This correlation is seen as evidence for increased sperm production driven by selection via sperm competition. However, also T levels may be under selection in scenarios of increased extra-pair paternity and as our study shows these T levels also correlate with testes size.

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SUMMARY

This study examines male and female influence on reproductive success in mallards (*Anas platyrhynchos*).

Chapter One investigates three notable features of the breeding system in wild mallard populations (Lake Starnberg and Lake Ammer, Southern Germany) based on microsatellite analysis of 41 clutches. First, adult populations are male biased, although mallards form social monogamous pairs and unpaired males may suffer reduced reproductive success. We show that this male surplus is already prevalent at egg laying (60% males). Second, egg dumping is a common female strategy in waterfowl and increases reproductive output of parasitic females. We report on high levels of brood parasitism in a mallard population with high nesting density (53%) whereas no egg dumping was observed under low nesting density. Finally, forced extra-pair copulations are commonly pursued by drakes. We assess the level of extra-pair paternity (56% of broods containing extra-pair young), which so far is the highest reported in waterfowl. However extra-pair fertilization was lower than expected from rates of extra-pair copulations described in literature.

Chapter Two experimentally examines the relevance of postcopulatory female control of male fertilization success in comparison to sperm competition. By artificially inseminating groups of four sisters with a sperm mixture containing equal sperm numbers of one brother and one unrelated male we did not observe any effect of parental relatedness on gain of paternity. However male reproductive success was significantly influenced by long-term sperm performance (sperm motility, sperm swimming speed).

Chapter Three investigates whether the female environment differentially influences sperm activity (concentration of motile sperm, sperm swimming speed). To test sperm activity in different female environment we measured sperm swimming in buffer and added female blood plasma. Again no effect of genetic relatedness was observed, but female reproductive status significantly influenced the amount of motile sperm and sperm

swimming speed. Furthermore we observed a strong individual female effect on sperm activity.

Chapter Four discusses the relationship between frequent copulations and ejaculate quality (sperm concentration, sperm swimming speed). After males were prevented to copulate with their social partner, sperm concentration and sperm velocity increased significantly. Therefore number of copulations trade against competitiveness of single ejaculates.

Chapter Five describes the positive relationship of testis size and circulating levels of testosterone in mallard drakes during the reproductive season.

CONCLUSION AND OUTLOOK

We found that extra-pair fertilizations in free-living mallards are less common than expected based on previously reported copulation frequencies. This could be the result of counteracting strategies of the social male (e.g. forced within-pair copulations) or be due to cryptic female choice. This study provides evidence that cryptic female sperm selection within the female reproductive tract purely based on sperm genotype (selection criterion: relatedness) is less important in determining paternity success than sperm competition. It remains to be investigated whether females can use additional cues obtained during natural matings to select sperm and/or whether they use other mechanisms to bias male fertilization success in their interest (e.g. sperm ejection, differential utilization of separated ejaculates). Moreover it remains to be evaluated to what extent selecting sperm via sperm competition *per se* is beneficial to female mallards (sexually selected sperm hypothesis).

This study highlights the importance of sperm quality (motility and swimming speed) for the gain of paternity in a competitive situation. However we also show that sperm swimming speed is not simply a male characteristic but is also shaped by the female environment. How this female-mediated effect affects male reproductive success and whether this can serve as a female mechanism to select sperm (based on other attributes than genetic relatedness) warrants further research.

Competitiveness of ejaculates (sperm concentration, swimming speed) was also influenced by the male reproductive behavior (copulation frequency). Potential alternative reproductive strategies of paired males (frequent copulations, reduced ejaculate quality) and unpaired drakes (rare copulations, superior ejaculate quality) may help to understand the paradoxical existence of a significant male surplus in primary and adult sex ratios in a socially monogamous species. Paternity analyses in wild populations will be necessary to further evaluate the existence and relative success of different reproductive strategies.

Finally we observed a positive correlation between levels of circulating testosterone (T) and testis size during the reproductive season. As shown for many species, testes size generally correlates with sperm production. Because females prefer to mate with males of higher spring T-levels during the pairing season in autumn, we suppose that females at the same time select males capable of higher sperm production.

APPENDIX

SEVEN POLYMORPHIC MICROSATELLITE LOCI FOR PATERNITY ASSESSMENT IN THE MALLARD (*ANAS PLATYRHYNCHOS* L.)

A. Denk, B. Gautschi, K. Carter & B. Kempenaers

ABSTRACT

We describe seven polymorphic microsatellite loci for the mallard (*Anas platyrhynchos*). The microsatellites presented here are highly polymorphic with on average 12 alleles in a sample of 15 presumably unrelated individuals. Therefore they enable detailed parentage analysis in wild mallard populations, and can be used to answer many intriguing questions in behavioral ecology and evolutionary biology.

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INTRODUCTION

Wild mallards recently became the target of growing interest in behavioral ecology and evolutionary biology for several reasons. First, in this seasonally sexually dimorphic species males exhibit conspicuous traits like the yellow bill, which became subject of recent observational and experimental studies. Omland (1996a; 1996b) showed that females base their mate preference on multiple male ornaments, in particular on the bill. Peters et al. (2004) correlated bill color with measures of immunocompetence and sperm quality, suggesting that it is an honest signal of male quality or condition. However, how these traits translate into actual reproductive success of male mallards is still unclear and deserves further study. The second remarkable feature of this species is that although mallards form stable socially monogamous pairs, females face frequent forced copulations by other males. This behavior is particularly interesting for studies on sperm competition and female strategies to bias paternity in their own interest (e.g. cryptic female sperm choice). However, only few data on parentage (e.g. levels of extra-pair paternity) in wild mallard populations are available yet (only one study, using 8 allozyme loci with a low general exclusion probability (p=0.539; Evarts and Williams 1987)). Finally, ducks are among those species where intra- and interspecific brood parasitism is common (Dugger and Blums 2001; Poysa 2003; Semel and Sherman 1992; Semel et al. 1988; Wilson 1990), but precise data for mallards are lacking. Studies on paternity and maternity greatly benefit from the use of microsatellite markers. These became available only very recently for the Peking duck, a domestic form of *Anas platyrhynchos* (Maak et al. 2000; Maak et al. 2003). However these markers show relatively little polymorphism (7 markers, number of alleles N_A=1-5, mean H_E=0.45, N=32 individuals, Maak et al. 2000; 18 markers, N_A=1-6, mean H_E=0.49, N=40 individuals, Maak et al. 2003), which restricts their use for parentage analysis. Here we report on the isolation of seven additional highly polymorphic microsatellite markers, which were developed particularly for parentage analysis in the wild mallard.

MATERIAL AND METHODS

A blood sample was collected from a single mallard, born in captivity from parents caught at Lake Starnberg (47º54'N/11º18'E), Southern Germany. The sample was stored in Queen's Lysis buffer (0.01 M Tris, 0.01 M NaCl, 0.01 M Na-EDTA, 1% *n*-Lauroylsarcosine; adjusted to pH8) and genomic DNA was extracted with the GFX™ Genomic Blood DNA Purification Kit (Amersham Pharmacia Biotech Inc., USA) according to the manufacturer's protocol. An enriched library was made by ECOGENICS GmbH (Zurich, Switzerland) from size-selected genomic DNA ligated into TSPAD-linker (Tenzer et al. 1999). The genomic library was enriched by magnetic bead selection with biotin-labelled $(CA)_{20}$ and $(GA)_{20}$ oligonucleotide repeats (Gautschi et al. 2000a; Gautschi et al. 2000b). Of 768 recombinant colonies, 133 gave a positive signal after hybridization. Plasmids of 37 clones were sequenced and primers were designed for 7 microsatellite loci, which were tested for polymorphism. We confirmed that none of the newly isolated microsatellites was identical to the loci isolated for the Peking duck previously (Maak et al. 2000; Maak et al. 2003).

We assayed variation among 15 presumably unrelated individual mallards, caught at 15 different locations at Lake Starnberg (approx. 65 km^2) and Lake Ammer (48º00'N/11º08'E; approx. 60 km^2), Southern Germany. Genomic DNA was extracted as described above. To assess the level of polymorphism we performed polymerase chain reaction (PCR) amplifications in a 10 µL reaction volume containing 10 ng genomic DNA, 100 µm dNTP, 0.12 µL of a 1% Bovine Serum Albumen, 1.5 mM $MgCl_2$, 10 mM 10 x Mg-free buffer (Promega), 0.5µM of each forward and reverse primer and 0.5 units of Taq DNA polymerase (Promega). Forward primers were labeled with fluorescent dyes (Apl2 – TET, Apl11 – NED, Apl12 – 6-FAM, Apl14 – TET, Apl23 – NED, Apl26 – 6-FAM, Apl36 – PET; Applied Biosystems). We used the following PCR profile on a GeneAmp® PCR System 2700 (Applied Biosystems): initial denaturation at 95 °C for 5 min followed by 30 cycles with 95°C for 30 sec, annealing temperature 60 °C (all primer pairs) for 30 sec and 72°C for 30 sec. After the last cycle, elongation at 72 °C was prolonged for 8 min. Amplified fragments were resolved on an ABI Prism 310 Genetic Analyzer (Applied Biosystems). Expected and observed heterozygosity were determined

and exact tests for departure from Hardy-Weinberg equilibrium (HWE) were performed using GENEPOP on the web (http://wbiomed.curtin.edu.au/genepop/).

RESULTS

All seven microsatellite loci show high levels of polymorphism (N_A=10-14, mean H_E=0.86, mean H_O=0.77, Table 1). Only one marker (Apl23) significantly deviated from HWE ($p<0.05$). Linkage could not be excluded for Apl11-Apl14, Apl12-Apl23 and Apl14-Apl23 ($0.001<p<0.05$). However**,** when the analysis was repeated with a different set of individuals (N=15), replacing nine individuals by their social partner, no linkage disequilibrium was observed ($p>0.05$). Across all seven loci the general exclusion probability is $p>0.999$ (Jamieson and Taylor 1997). Even if only one parent is known, the exclusion probability remains high ($p=0.998$). Thus, the highly polymorphic loci presented here substantially expand the available marker set developed for the domestic Peking duck (Maak et al. 2000; Maak et al. 2003) and should be valuable for applications in population genetic studies, and in particular for studies on paternity (e.g. extra-pair paternity studies) and maternity (brood parasitism).

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LOCUS	Repeat motif based on sequence clone	Primer sequence (5'-3')	T_a (ºC)	No. of alleles	Size-Range (bp)	H_O	H_E	Accession no.
Apl2	$(CA)_{15}GA(CA)_{32}$ $AAA(CAA)_4$	F: GATTCAACCTTAGCTATCAGTCTCC R: CGCTCTTGGCAAATGTCC	60	12	96-140	0.93	0.90	AY498540
Apl11	$(GA)_{25}$	F: AACTACAGGGCACCTTATTTCC R: TTGCATCAGGGTCTGTATTTTC	60	13	92-136	0.87	0.87	AY498541
Apl12	$(GA)_{27}$	F: AGTTGACCCTAATGTCAGCATC R: AAGAGACACTGAGAAGTGCTATTG	60	10	112-155	0.73	0.80	AY498542
Apl14	$(CA)_6CG(CA)_4TA$ $(CA)_4TA(CA)_{10}$	F: CCTTTTCCAAGGGGCTACAC R: CTCTTCCCCAAAACGTCATC	60	14	117-175	0.87	0.90	AY498543
Apl23	$(TG)_{13}(TC)_3(TG)_2TC$ $CG(TG)_3TCTN(TG)_7$ $CG(TG)_2(TC)_3TG$	F: GAAGAGGCAGTGGCAACG R: GCTGAGATGCTCCCAGGAC	60	11	141-265	0.53	0.84	AY498544
Apl26	$(CA)_{11}(GA)_9$	F: AACAGGGATAACATGAGAAGTGG R: TGAGCAGCTGTCTGGTATCTATTC	60	11	138-156	0.73	0.88	AY498545
Apl36	$(CA)_{13}GA(CA)_3(GA)_2(CA)_2GA(CA)_{10}GA$ $(CA)_7GA(CA)_2TA$ $(CA)_5$	F: ATGCTTTGCTGTTGGAGAGC R: TCCACTGGGTGCAAACAAG	60	13	146-229	0.73	0.85	AY498546

F, forward primer; R, reverse primer; T_a, optimal annealing temperature; H_O, observed heterozygosity; H_E, expected heterozygosity.

Table 1. Characterization of seven microsatellite loci from *Anas platyrhynchos* (N=15 individuals genotyped)

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writing the acknowledgment and enjoying to thank all the people, who helped me to proceed with my work and who made this a very special and valuable time of my life.

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ERKLÄRUNG

Hiermit versichere ich, dass ich die vorliegende Arbeit selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.