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Seasonal variation in ejaculate traits of male red-winged blackbirds (*Agelaius phoeniceus*)

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Abstract Many reproductive traits, including ejaculate characteristics, usually show remarkable seasonal variation, but the potential for such dynamics in sperm morphology has been overlooked. Several studies have revealed high within-male repeatability in sperm morphology, but samples have typically been collected within a few hours or days, and the consistency of sperm morphology over longer periods remains unexplored. Here, we tested whether ejaculate traits, including sperm morphology, sperm number, and sperm velocity, exhibit seasonal phenotypic plasticity in a long-lived seasonal breeder, the red-winged blackbird (*Agelaius phoeniceus*). We found absolute and/or relative flagellum length and sperm velocity to increase across the season, whereas sperm numbers within ejaculates declined. Sperm morphological traits were further positively associated with harem size or the total number of offspring that fledged in each male's territory, suggesting that sperm morphology is likely to be linked to male reproductive quality. The underlying mechanisms of these patterns of seasonal variation remain unresolved, but we discuss our results in the context of dynamics of reproductive hormones, testicular structures and function, and reproductive behavior.

Keywords Sperm morphology · Sperm velocity · Sperm number · Seasonal variation · Sperm competition · Phenotypic plasticity

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Introduction

Sperm size and shape vary considerably across taxa (Cohen 1977; Jamieson 2007; Pitnick et al. 2009). The causes and consequences of this variation, however, are still poorly understood, in particular for intra-male variation (e.g., Ward 2000; Pitnick et al. 2009). Sperm morphology generally varies little within males compared to that between males (Woolley 1971; Ward 2000; Morrow and Gage 2001a; Simmons and Kotiaho 2002; Birkhead et al. 2005). Whereas there is ample evidence that sperm morphology can respond rapidly to selection over few generations (Woolley 1971; Morrow and Gage 2001a; LaMunyon and Ward 2002; Miller and Pitnick 2002; Dobler and Hosken 2010), recent work indicates that it can also vary within generations in response to differences in the males' social environment that may influence the likelihood of competitive fertilization (Crean and Marshall 2008; Immler et al. 2010). In addition to plasticity in relation to the environment, an age-dependent increase in sperm length has been reported within individual rove beetles (*Aleochara bilineata*; Green 2003). Apart from these few examples, however, intra-male variation in sperm morphology in response to varying ecological or life-history traits remains largely unexplored. This is partly due to only few studies measuring sperm morphological traits in more than a single ejaculate of

individual males and, if they have, the typically short period (a few hours or days) between repeated samples may have rendered such effects difficult to detect, given the time required to produce a new generation of sperm (e.g., Birkhead and Fletcher 1995; Morrow and Gage 2001b; Simmons et al. 2003; Harris et al. 2007).

Seasonal breeders such as most temperate birds provide an interesting case for studying dynamics in sperm traits both within and between breeding seasons. First, intra- and inter-seasonal fluctuations in the availability of fertile females may result in varying levels of extra-pair copulations and thus varying degrees of postcopulatory sexual selection (e.g., Westneat and Gray 1998), which could result in phenotypically plastic responses in male ejaculate investments. Secondly, seasonal breeders typically undergo an annual cycle of recrudescence and regression of the testes (Wright and Wright 1944; Selander and Hauser 1965; Partecke et al. 2004; Calhim and Birkhead 2007). Across species, sperm length covaries strongly with the diameter of the seminiferous (i.e., sperm-producing) tubules within the testes (Lüpold et al. 2009c), which themselves follow the annual recrudescence and regression of the testes (e.g., Wright and Wright 1944; Selander and Hauser 1965; Partecke et al. 2004). Associated seasonal variation in sperm traits remains unexplored, but a recent study showing a rapid response in sperm morphology to experimentally manipulated sperm competition risk in the Gouldian finch (*Erythrura gouldiae*; Immler et al. 2010) suggests that there may be greater potential for plasticity than has previously been assumed.

Here, we examined the variation in sperm morphology both within and between males from natural populations of red-winged blackbirds (*Agelaius phoeniceus*). In this species, sperm morphology varies significantly between geographically distinct populations (Lüpold et al. 2011a), but less is known about variation between and within individuals of the same population. Compared to the zebra finch (*Taeniopygia guttata*), for which within- and between-male variation has been described (Birkhead and Fletcher 1995; Birkhead et al. 2005; Immler et al. 2012), the red-winged blackbird exhibits a much higher degree of sperm competition and much less variation in sperm morphology both between and within males (Calhim et al. 2007; Immler et al. 2008). Further, red-winged blackbirds are seasonal breeders (e.g., Beletsky 1996) and thus more likely to show phenotypic plasticity or temporal variation in sperm traits than zebra finches, which are opportunistic, non-seasonal breeders that, under domestication, typically breed continuously (Farner and Serventy 1960; Sossinka 1982).

We measured sperm morphology in repeated ejaculates from individual male red-winged blackbirds across the breeding season in three populations and, in one of them, across two consecutive years. In the same population, we also examined seasonal variation in sperm velocity and the number of sperm contained in ejaculates in one of the years. Both these ejaculate variables are important predictors of male fitness in situations of sperm competition, with fast and numerous sperm typically being advantageous (e.g., Martin et al. 1974; Birkhead et al. 1999). Additionally, they might both be linked to sperm morphology, because across different taxa, sperm velocity has been shown to increase with absolute and relative flagellum length as the "engine" of the sperm (e.g., Gomendio and Roldan 2008; Humphries et al. 2008; Fitzpatrick et al. 2009; Lüpold et al. 2009a), with a genetic link between these traits demonstrated in the zebra finch (Mossman et al. 2009). Similarly, sperm number may be traded off against sperm length due to energetic, functional, or spatial constraints during sperm production (Pitnick 1996; Oppliger et al. 1998; Lüpold et al. 2009c; Ramm and Stockley 2010; Immler et al. 2011).

Material and methods

Study sites and sample collection

We analyzed sperm samples that were collected as part of several research projects in three wild populations of red-winged blackbirds. Most samples were collected as natural ejaculates (in 2005) or from fecal samples (in 2006, see below) near the Kellogg Biological Station in Hickory Corners, Michigan, USA (henceforth "MI"). The other two populations were near Bismarck, North Dakota ("ND"), where sperm were collected from dissected birds in 2006 (Lüpold et al. 2009c), and near Drakesboro, Kentucky ("KY"), where natural ejaculates were collected in 1996 (Westneat et al. 1998).

The MI population was subdivided into relatively small patches of marsh within 0.5–8 km of one another, each occupied by 9–22 territorial males. The distance between sites was far below the reported mean dispersal distance (Dolbeer 1978), and marked birds were occasionally sighted between different sites, so we considered them to belong to one large population. On the two sites with most males, we marked all individuals with unique combinations of a numbered and three colored leg bands at the beginning of each season. Most of the small sites were on private land where we obtained permission to work for a day in 2005 and thus were unable to mark the birds. However, due to relatively small numbers of territories, typically located sequentially in strip marshes, we were able to identify territorial males by observing their movements before collecting samples.

In 2005 (MI), we collected natural ejaculates using model females mounted in copulatory posture onto poles and fitted with a false cloaca, onto which males deposited their ejaculate during copulation (Pellatt and Birkhead 1994;

Westneat et al. 1998). Immediately after copulation, we collected the ejaculate with a Gilson pipette and diluted it in 400 μl of Dulbecco's modified Eagle medium (DMEM; Invitrogen, Ltd.), and, within 7.1±0.28 min (mean±SE; range 3–11 min) of copulation, placed 15 μl of the well-mixed sample under a phase-contrast microscope at 35.0± 0.1 °C and video-recorded it at a magnification of×200 for measurements of sperm velocity using computer-aided sperm analysis (Hobson Tracking Systems Ltd., U.K.; for further details, see Lüpold et al. 2009b). The remainder of each diluted sample was fixed by adding 20 μl of formalin to a final concentration of 5 %.

Although natural ejaculates are essential for quantifying the sperm delivered during copulation, the use of model females is often not successful for collecting multiple samples from individual males because males can habituate to the models quickly (D. F. Westneat and S. Lüpold, personal observation). In 2006, when we mainly focused on collecting repeated samples from individual males across the season for intra-male variation in sperm morphology, we thus established a wooden platform (ca. 20×30 cm) on a pole in each of the 22 male territories of our largest site and covered it with plastic. Males preferentially perched on these platforms as they projected above the surrounding cattails (*Typha latifolia*). We observed focal males (all individually marked) until they flew off the platform, immediately examined the plastic surface for fresh feces, picked up the wet part with a pipette, and placed it in 5 % formalin solution (diluted in DMEM) before wiping off the remaining fecal matter. Feces of breeding male birds often contain sperm and provide a simple and non-invasive technique for sperm collection (Immler and Birkhead 2005). We minimized the risk of mistaking fecal samples with those from non-focal males by placing the platforms close to the center of the respective territories, thoroughly cleaning the surface regularly, and focusing on males landing on platforms that had not been visited by other birds since cleaning.

Ejaculate analyses

For sperm counts in ejaculates (MI in 2005 and KY), we determined the total volume of the samples to the nearest microliter using a Gilson pipette, quantified the sperm using an Improved Neubauer Chamber, and, for MI (2005), corrected all sperm counts for the 15 μl removed for analysis of sperm velocity, assuming equal sperm concentration in all well-mixed subsamples. We performed two counts for all MI samples, with an intra-sample repeatability of 0.96 ($F_{147,148}$=43.6, P<0.0001) following Nakagawa and Schielzeth (2010).

Using computer-assisted image analysis, we determined (to the nearest 0.1 μm) the respective lengths of the midpiece and flagellum, and the flagellum/head and midpiece/flagellum ratios for ten sperm per sample. Ten sperm per sample are representative for calculations of sample means of sperm dimensions in passerine birds due to high consistency within ejaculates (online Supplementary Fig. S1; also see Birkhead et al. 2005; Calhim et al. 2007, 2011; Immler et al. 2008). Absolute and relative measures of midpiece and flagellum length have previously been shown to covary positively with sperm velocity (e.g., Lüpold et al. 2009a; Mossman et al. 2009; Laskemoen et al. 2010; but see Lüpold et al. 2009b) and are thus likely to be important in situations of sperm competition. Whereas the flagellum/head ratio accounts for the drag of the head and opposing propulsive force of the flagellum (Higdon 1979; Humphries et al. 2008), the midpiece/flagellum ratio reflects a possible indicator of the metabolic regulation of sperm, with the midpiece providing the energy for the flagellar activity (Cardullo and Baltz 1991). Since in the red-winged blackbird the flagellum accounts for approximately 90 % of the total sperm length and is tightly correlated (R^2=0.94, t= 51.41, P<0.0001, N=179), we omitted total sperm length as a separate sperm trait.

Nesting data

In the MI (2005) population, we mapped the territories of marked males and surveyed all nests therein every 1–3 days for respective dates of nest building, egg laying, hatching and fledging, and respective numbers of eggs, hatchlings, and fledglings (N=97 nests). Further, we marked the females attending each nest with unique combinations of colored leg bands. In the current study, we focused on the number of potentially fertilizable females (i.e., 8 days before first egg until 2 days after last egg; Westneat 1993b) at the population level as an estimate of nesting synchrony and the potential risk of sperm competition at the time of ejaculate collection.

Statistical analyses

All analyses were conducted using the software package R (version 2.12.2; R Development Core Team 2011). In the Michigan population, we examined seasonal variation in ejaculate traits (including their potential response to variation in nesting behavior) using a reaction norm approach (e.g., Woltereck 1909; Stearns 1989; Schlichting and Pigliucci 1998; Nussey et al. 2007). Date can be considered a continuous environmental variable underlying seasonal variation in numerous reproductive traits that may affect ejaculates, including testis size, breeding stage, availability of fertile females, or male condition and physiology. Following this approach, the sperm morphology×date reaction norm can vary in both its elevation (e.g., a male's expected sperm morphology on the average day of the

season) and slope (i.e., extent of a male's response to seasonally changing variables). In linear mixed-effects models (LME) with restricted maximum-likelihood parameter estimation (REML) and male identity as the random factor, we analyzed between-individual variance by centering individual means about the grand mean of sampling date ("MCMDate," (grand) mean-centered mean date) and within-individual variance using values centered about within-subject means ("IMCDate," individually mean-centered date; Nussey et al. 2007; van de Pol and Wright 2009). Biologically, MCMDate could capture either phenotypic plasticity or some bias in which males were sampled, whereas IMCDate would capture the intra-male degree of phenotypic plasticity (Nussey et al. 2007).

In the analyses on sperm velocity, we further controlled for the time between ejaculation and the start of recordings as sperm velocity in passerine birds has previously been shown to decline relatively rapidly over time (e.g., Helfenstein et al. 2008, 2010). In the analyses on sperm number, we controlled for the time of day that samples were collected because ejaculate size might decline across a day as the number of previous copulations increases, even though red-winged blackbirds apparently replenish their sperm reserves relatively rapidly (Westneat et al. 1998). Analogous to the date variable, we calculated time values centered about the grand mean and individual mean ("MCMTime" and "IMCTime," respectively). For the 2005 season, when males from different sites were sampled, we further nested males within sites in the random factor. Finally, we accounted for potential quadratic seasonal variation in ejaculate traits (at the among-male level) as they might occur in association with the seasonal patterns of testis size, for example, but since the quadratic date term was not significant in any of the models and all models improved by excluding this term (based on AIC scores), we will only report models without quadratic terms.

To test whether variation in sperm morphology was related to changes in the social circumstances, in particular the availability of fertilizable females, we used the respective number of potentially fertilizable females at the population level as the explanatory variable and both MCMDate and IMCDate as covariates.

Results

Intra- and inter-male variation in sperm morphology

In the MI population, we collected 176 ejaculates from 130 males in 2005 and 286 fecal samples from 38 males in 2006. Of the fecal samples, 109 (38 %) contained at least ten morphologically intact sperm for measurements, resulting in 2.9±0.36 (mean±SE) successful samples per male. Within samples, sperm dimensions were highly consistent, with as few as two sperm capturing >90 % of the variation for most traits (online Supplementary Fig. S1) and within-sample coefficients of variation averaging 1.85±0.07 % for midpiece length, 1.47±0.07 % for flagellum length, 5.15±0.20 % for the flagellum/head ratio and 2.02±0.06 % for the midpiece/flagellum ratio among all natural ejaculates and 1.76±0.05, 1.66±0.07, 5.45±0.31, and 1.68±0.07 %, respectively, among the fecal sperm samples.

An LME model (REML) controlling for date of sample collection revealed significant repeatability (i.e., random factor variance components; Nakagawa and Schielzeth 2010) for the dimensions of different sperm components across years within the males with sperm collected in both 2005 and 2006 (N=85 samples from 17 males; midpiece, repeatability R=0.32, P=0.0001; flagellum, R=0.29, P=0.001; flagellum/head ratio, R=0.21, P=0.05; midpiece/flagellum ratio, R=0.38, P<0.0001). Consistently, we found no significant difference between years for any of the sperm morphological traits (LME; flagellum/head ratio, $F_{1,66}$=2.97, P=0.09; all other traits, $F_{1,66}$<0.92, all P>0.34), which also suggests that the different sampling methods between years did not significantly affect sperm measurements.

Seasonal effects on sperm morphology

We tested for intra-seasonal changes in sperm morphology both at the population level and within individual males. In MI (2005), we collected samples between 18 April and 29 June. It seems likely that this 10-week period spanned the entire period that copulations occurred, given that eggs were laid from 26 April to 24 June on our main study site in that year. In this year, the absolute sperm dimensions increased with MCMDate and with ICMDate, whereas the flagellum/head ratio increased only with ICMDate and the midpiece/flagellum ratio showed no directional seasonal variation (Table 1 and Fig. 1). In MI (2006), when fecal samples were collected between 12 April and 17 July, we found an increase in the flagellum/head ratio across the season at both the inter-male and intra-male levels, while flagellum length increased only within males, and both absolute and relative midpiece length were not associated with date at both levels (Table 1).

We did not have comparable data for the ND and KY populations because, in ND, we collected samples from dissected males, and in KY, repeated samples of each male were taken on the same day (see Westneat et al. 1998). However, having identified similar temporal patterns in sperm morphology both within and between males in the MI population (particularly increase in flagellum length), the other two populations allowed us to establish whether these reflected more general patterns, at least at the population level.

Table 1 Summary of linear mixed models (REML) testing for seasonal effects (expressed by MCMDate and IMCDate) on sperm morphology in the MI population, with male as the random effect (2005, N=176 samples from 130 males; 2006, N=109 samples from 38 males)

	Location	Male		Mean date (MCMDate)		Within-male date (IMCDate)	
Trait	2005	2005	2006	2005	2006	2005	2006
Midpiece	0.12±0.09	9.2±1.6	4.6±1.6	**0.11±0.02**	0.02±0.02	**0.07±0.03**	0.01±0.01
	8.2 (<0.01)	25.7 (<0.01)	30.0 (<0.001)	**31.2 (<0.001)**	1.6 (0.21)	**4.7 (0.036)**	1.6 (0.21)
Flagellum	2.7±1.7	10.2±1.4	1.0±0.6	**0.09±0.02**	0.01±0.02	**0.04±0.02**	**0.03±0.01**
	17.1 (<0.001)	85.8 (<0.001)	12.7 (0.005)	**26.2 (<0.001)**	0.6 (0.45)	**8.4 (0.006)**	**8.1 (0.006)**
F/H ratio	0.04±0.02	0.10±0.03	0.07±0.03	0.005±0.003	**0.01±0.005**	**0.01±0.005**	**0.03±0.01**
	15.1 (0.002)	7.56 (0.006)	4.3 (0.04)	1.39 (0.24)	**9.1 (0.005)**	**2.5 (0.015)**	**9.3 (0.003)**
M/F ratio	<0.01±0.00	<0.01±0.00	<0.01±0.00	−0.0001±0.0001	0.0002±0.0002	–0.0002±0.0002	–0.0001±0.0001
	24.8 (<0.001)	21.4 (<0.001)	26.8 (<0.001)	1.1 (0.29)	1.0 (0.32)	1.2 (0.29)	2.2 (0.14)

For each trait, the variance estimates ± SE (location and male) or slopes ± SE (date terms) are presented in the top row and the statistic (−2 Δlog-likelihood, *LL*, for location and male or *F* for both date terms) in the bottom row, along with the *P* value in parentheses. In the 2005 data, male was nested within location due to multiple sampling locations. In the 2006 data, where most males had repeated samples, allowing slopes to vary among males in addition to intercepts improved the model fit only for flagellum length (flagellum, LL=5.64, P=0.02; all other traits, LL<0.28, P>0.60). Statistically significant date effects are highlighted in bold

In 2005, males with 1, 2, or >2 samples each (max. nine samples/male): N=102, 19, and 10, respectively; df (MCMDate)=1,115; df (IMCDate)=1,45

In 2006, males with 1, 2, or >2 samples each (max. nine samples/male): N=14, 7, and 17, respectively; df (MCMDate)=1,36; df (IMCDate)=1,70

F/*H ratio* flagellum/head ratio, *M*/*F ratio* midpiece/flagellum ratio

In ND, we measured sperm from 32 males dissected immediately after death in two brief periods between 16 May and 12 June 2006, with 24 days between collecting sessions. Across these two time points, we found significant increases in flagellum length (mean±SE=129.1±0.5 μm (N=16) versus 131.3±0.6 μm (N=16); t=2.83, P=0.009) and the flagellum/head ratio (8.01±0.05 versus 8.57±0.12; t=4.31, P=0.0004), whereas midpiece length did not change (108.1±0.8 μm versus 107.5±1.1 μm; t=–0.91, P=0.37). Combined with the increase in flagellum length, this resulted in a decrease in the midpiece/flagellum ratio (0.90±0.004 vs. 0.88±0.004; t=−3.00, P=0.005).

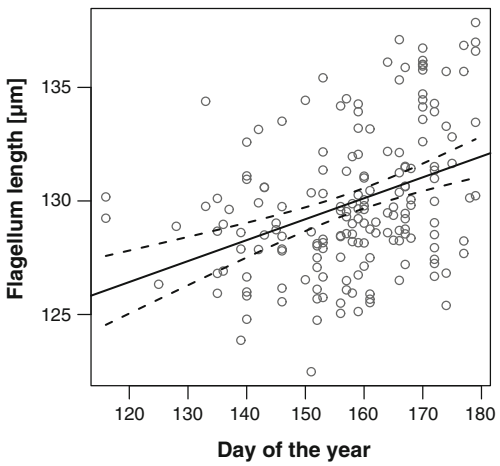

Fig. 1 Positive relationship with 95 % confidence interval between flagellum length and day of the year in the Michigan population of 2005. For simplicity, data are shown as raw values with each point representing an individual sample (N=169, r=0.37, P<0.0001). For controlled results using MCMDate and IMCDate, see Table 1

In the KY population, we analyzed 56 ejaculates taken from 22 different males (2.5±0.4 samples/male) between 25 April and 14 June 1996. In LME models on these males, we found an increase with MCMDate in midpiece length (slope b±SE=0.11±0.03; $F_{1,33}$=15.03, P=0.0005), flagellum length (b=0.13±0.03; $F_{1,33}$=22.29, P<0.0001), and the midpiece/flagellum ratio (b=0.0004±0.0001; $F_{1,33}$=5.90, P=0.021), but not in the flagellum/head ratio (b=−0.002±0.005; $F_{1,33}$=0.09, P=0.77).

Seasonal effects on sperm number and velocity

For the ejaculates collected in MI (2005), we also examined temporal effects on sperm number and sperm velocity. We conducted the same statistical analyses as described above, but additionally corrected for the time of day that samples were collected for sperm counts (see "Material and Methods"). Due to lumps of sperm in some samples and because some ejaculates could not be collected completely (e.g., in feathers next to false cloaca), our sample size for sperm counts was reduced to 146 ejaculates from 114 different males. Total sperm number per ejaculate decreased with the time of day within males (IMCTime; b=−33.4±16.3, $F_{1,29}$=4.19, P=0.05) but not across males (MCMTime; b=3.17±3.43, $F_{1,29}$=0.85, P=0.36), and it decreased across the season with MCMDate (b=−0.09±0.04, $F_{1,99}$=7.15, P=0.01) and with IMCDate (b=−0.22±0.10, $F_{1,29}$=5.0, P=0.03; Fig. 2a). Although the decrease in

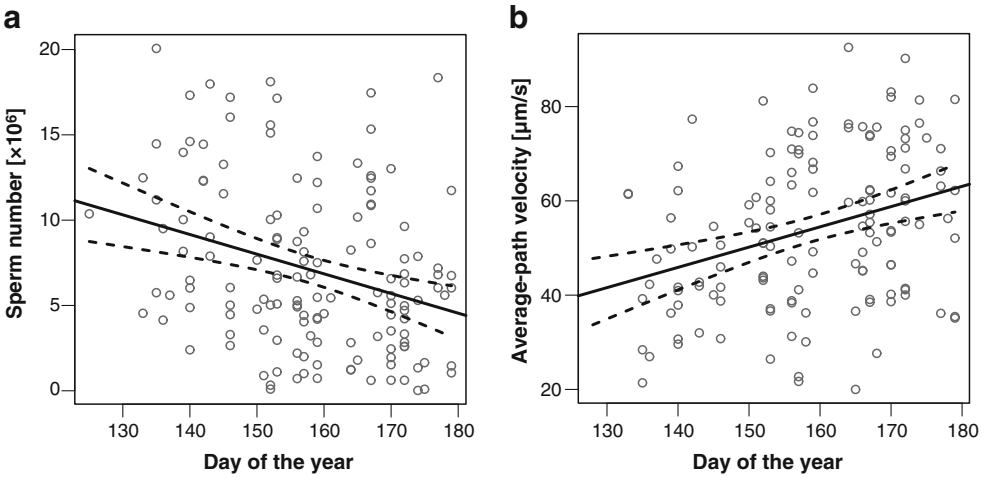

Fig. 2 Seasonal change in **a** sperm number (N=140, r=−0.31, P=0.0002) and **b** average path velocity in natural ejaculates (N=136, r=0.33, P<0.0001) of the population in Michigan (2005). For simplicity, data are shown as raw values with each point representing an individual sample. For controlled results using MCMDate and IMCDate, see main text. *Dashed lines* represent 95 % confidence intervals

sperm number combined with the increase in flagellum length over time may suggest a trade-off between sperm size and number within ejaculates, we found no effect of sperm length as an additional predictor in the above model ($F_{1,28}$=0.13, P=0.73; but no qualitative change in date variables, P≤0.03) or in a simple model without time variables ($F_{1,31}$=0.05, P=0.82).

In the 135 samples from 107 males of the MI population (2005), for which we successfully obtained sperm velocity data, and controlling for the time between ejaculation and the start of the recording (log-transformed; b=−5.12±2.55, $F_{1,26}$=3.87, P=0.06), average path velocity (i.e., smoothed and averaged sperm trajectory) increased across the season with MCMDate (b=0.37±0.13, $F_{1,92}$=7.62, P=0.007; Fig. 2b) and, albeit not significantly, with IMCDate (b=0.48±0.29, $F_{1,26}$=2.84, P=0.10). The increase over time in both sperm velocity and flagellum length may suggest that the concerted seasonal changes of these two sperm traits may be due to a direct (e.g., genetic) positive correlation between them. However, we found no significant effect of flagellum length on sperm velocity ($F_{1,25}$=0.54, P=0.47) in a model controlling for MCMDate, IMCDate, and the time to recording, all of which did not change qualitatively compared to the above analysis on sperm velocity alone. We also obtained qualitatively similar results when using other absolute or relative sperm dimensions as measures of sperm morphology or collated different velocity parameters in a principal components analysis (also see Lüpold et al. 2009b).

Ejaculate traits relative to fertile females and reproductive success

For the samples from MI (2005), we tested whether variation in ejaculate traits was associated with the number of simultaneously fertile females on the primary study site, which peaked between 7 and 14 May with 48–49 females and declined to less than ten females within the subsequent 2 weeks to peter out during the remainder of the season. Controlling for MCMDate and IMCDate, we found no significant associations between the number of fertile females and any of the morphological sperm traits (all $F_{1,44}$>0.35, all P>0.55), sperm count ($F_{1,31}$=0.70, all P=0.41), or sperm velocity ($F_{1,25}$=0.49, all P=0.49), whereas both date effects did not differ qualitatively from those in Table 1.

On the largest site of the same population, we also monitored all nests (N=97 among 22 male territories). Based on trait means for each male with complete nesting and sperm morphological data (N=20 males), midpiece length, flagellum, and the flagellum/head ratio were all positively associated with harem size (i.e., the maximum number of simultaneously nesting females, ranging between 1 and 8 females; t>2.15, P<0.046) or the total number of offspring that fledged in each male's territory (t>2.66, P<0.016). Midpiece length and the flagellum/head ratio were additionally positively correlated with the mean clutch size among all nests on their territories, which ranged between 2.5 and 4.0 eggs (t>3.60, P<0.002). The midpiece/flagellum ratio, however, was not associated with any breeding data (all P>0.14). Similarly, sperm velocity did not covary with any breeding data (all P>0.15). These indicate that males that are superior at acquiring mates and likely to be superior social mates (based on nesting success) may also produce sperm with a relatively longer flagellum. It now needs to be established whether this variation in sperm morphology is also directly linked to fertilization success after controlling for extra-pair fertilization to obtain a more complete picture of potential fitness consequences. Due to incomplete paternity data, we were not able to test this idea empirically.

Discussion

In red-winged blackbirds, we found sperm morphology to be highly consistent within ejaculates but to significantly

vary across the breeding season. In particular, flagellum length increased over time in three separate populations. In one of these populations, flagellum length, and the flagellum/head ratio increased significantly within males in each of two different years examined. Additionally, albeit in just 1 year and population, the number of sperm per ejaculate decreased across the season, whereas sperm velocity tended to increase. Since both sperm morphology and sperm velocity have been shown to covary with reproductive success in birds (e.g., Birkhead et al. 1999; Denk et al. 2005; Laskemoen et al. 2010; Calhim et al. 2011), it appears that overall sperm quality may increase over the season while sperm quantity declines. The underlying mechanisms for these seasonal trends are unclear, but we propose potential explanations and encourage further research for a better understanding on these seasonal patterns, including investigations targeting their potential adaptive significance.

Seasonal variation in ejaculate traits

Our analyses revealed consistent sperm morphology within individual ejaculates but a significant increase across the breeding season, in particular for flagellum length. These results are consistent with a recent study in the house wren (*Troglodytes aedon*), which documented a similar increase in the flagellum/head ratio across the breeding season (Cramer et al. 2012). Although Cramer et al.'s (2012) study is based on inter-male variation, thereby hampering conclusions about intra-male phenotypic plasticity, the similarity between our results in two phylogenetically distinct species indicates a potential for such seasonal variation in absolute or relative flagellum length to be a more widespread phenomenon in seasonally breeding passerines, but further investigations are clearly warranted.

One potential parameter driving the seasonal variation in sperm morphology is the well-established seasonal change in the testosterone titer. For example, the plasma testosterone level of red-winged blackbirds shows considerable seasonal changes across the different reproductive stages, with a typical peak around female initiation of nesting and a gradual decline thereafter (Beletsky et al. 1989; Johnsen 1998). (Johnsen 1998) also reported a net decrease in plasma testosterone across the season at the population level, possibly because the initial peak is largely synchronous between males but increasing numbers of males return to lower levels of testosterone as the season progresses. In synergy with the follicle-stimulating hormone, testosterone promotes sperm production, including the activity of Leydig and Sertoli cells and consequently the spermatogenic capacity and sperm maturation (Wingfield and Moore 1987; McLachlan et al. 1995, 1996; Kirby and Froman 2000). Immler et al. (2010) showed that testosterone levels covary with flagellum or midpiece length in socially dominant Gouldian finches in response to changes in the social environment, although a direct causal link needs to be established. In this context, it seems plausible that fluctuations in testosterone and other hormone titers across the season may also influence ejaculate traits in the red-winged blackbird, potentially mediated through dynamics in spermatogenesis.

Whether influenced by hormones or not, a potential link between spermatogenesis and sperm morphology might be found in the sperm-producing testis structures. For example, across passerine birds, sperm length is positively associated with the diameter of the seminiferous tubules (Lüpold et al. 2009c), and also covaries with spermatogenic function of the seminiferous epithelium (Ramm and Stockley 2010; Lüpold et al. 2011b). As in other seasonally breeding temperate passerines, testis mass of the red-winged blackbird varies almost 500-fold across the annual cycle and covaries positively with the diameter of the seminiferous tubules (Wright and Wright 1944). The precise dynamics of these tubules across the breeding season are poorly understood, but if sperm morphology is also associated with seminiferous tubule size within species, it may vary with seasonal fluctuations in testis architecture. Among the 12 males in our recent study on testis histology (Lüpold et al. 2009c), we found no direct relationship between mean sperm length and mean tubule diameter. However, across 11 male brown-headed cowbirds (*Molothrus ater*), in which the inter-male variation in sperm length is substantially greater than in the red-winged blackbird (Lüpold et al. 2009b), rendering a relationship more easily detectable, mean sperm length was positively associated with the mean diameter of the seminiferous tubules (S. Lüpold, unpublished data). Consequently, an effect of testis size or structure on sperm length (and thus possibly sperm number) may exist within some species, but we need further data to draw firm conclusions.

Similar to testis structures, it is also possible that the kinetics of sperm production varies seasonally, potentially under the influence of changing androgen levels (see above). In mammals, the duration of spermatogenic cycle increases with sperm length (Ramm and Stockley 2010), but we do not know if this also the case in birds and to what extent the spermatogenic rate varies intra-specifically, particularly in the context of seasonal variation in ejaculate characteristics.

Given the growth and regression of the testes across the season, the question arises why we found a linear rather than quadratic relationship between flagellum length and date. One possible explanation is that the seminiferous tubules may not reach their maximal size until advanced stages of the egg-laying period and regress asynchronously among males (Wright and Wright 1944), thereby potentially resulting in the appearance of an overall linear increase in the population even if there was a quadratic relationship within

males. Our data do not permit any formal analysis of quadratic intra-male relationships due to small numbers of repeated samples per male. Similarly, our data allowing comparisons among males late in the season are likely to be biased towards males that were still in full breeding condition because we discontinued collecting sperm shortly after the last eggs were laid, such that the samples collected successfully late in the season may have been from males whose testes had not yet begun to regress. Furthermore, the completion of a single generation of sperm can take several weeks, such that the collected sperm would have initiated sperm elongation several days before the time of sperm collection, even if the testes may have begun to regress by that time. Information on the duration of the spermatogenic cycle is lacking for passerine birds, but in a handful of domestic galliforms studied so far, it takes 13–16 days (e.g., Clulow and Jones 1982; de Reviers 1988). With an expectedly lower male copulation frequency towards the end of the season (due to fewer fertile females available), sperm may additionally have been stored in the seminal glomera for several days before collection (Wolfson 1954).

We also found different morphological traits of sperm to vary somewhat independently. For example, the flagellum elongated over time in all three populations, but the midpiece did so only in KY and in the 2005 dataset of MI, thereby also resulting in different directions of seasonal changes in the midpiece/flagellum ratio among populations. Although the midpiece and flagellum can be phenotypically correlated (r=0.56 in the present dataset) and genetically linked (e.g., Birkhead et al. 2005), previous studies have shown that they can vary independently within or between males (e.g., Woolley 1971; Birkhead et al. 2005; Immler et al. 2010). Whether different seasonal trajectories in sperm dynamics between the three populations studied are associated with overall differences in sperm morphology (Lüpold et al. 2011a) remains to be determined.

Seasonal variation in the other ejaculate traits may have a similar proximate explanation as for sperm morphology. For example, if testosterone promotes sperm production, including the spermatogenic capacity (see above), the gradual decline in testosterone across the season might explain the observed decrease in sperm quantity from the early to the late season. Similarly, sperm behavior can be affected by the composition of seminal fluid (e.g., Cornwallis and O'Connor 2009), which in turn can fluctuate seasonally or be directly influenced by testosterone (e.g., McDowell et al. 1996; Matsuoka et al. 2006). Both these ejaculate characteristics can thus vary independently of each other or of sperm morphology, and it remains to be established how they might be functionally linked.

Overall, our data show significant variation in ejaculate characteristics across the breeding season similar to the house wren (Cramer et al. 2012), suggesting that seasonal breeders may be more susceptible to temporal effects than non-seasonal species like the zebra finch (Birkhead and Fletcher 1995; Zann 1996). Systematic examination of sperm morphology and other ejaculate traits throughout the breeding season in other seasonal and continuous breeders might help us elucidate the factors determining sperm variation within and between males.

Is seasonal variation in ejaculate traits adaptive?

The observed seasonal variation in ejaculate traits raises the question of whether it is adaptively significant. For example, the flagellum is the engine of the sperm, and sperm with a relatively longer flagellum may swim faster (e.g., Humphries et al. 2008; Lüpold et al. 2009a; Mossman et al. 2009), thereby exhibiting a reproductive advantage in competitive fertilizations (Birkhead et al. 1999; Gage et al. 2004; Denk et al. 2005; Gasparini et al. 2010; Boschetto et al. 2011; but see Dziminski et al. 2009). Thus, the seasonal increase in flagellum length could be the result of absolutely or relatively long flagella being advantageous but constrained by, or traded off against, other traits during the early season (e.g., seminiferous tubule size or spermatogenic function). We did not detect a direct association between sperm morphology and sperm velocity after controlling for the day of season (also see Lüpold et al. 2009b), but this does not necessarily preclude the possibility that a link may exist as it does across passerine birds (Lüpold et al. 2009a; but see Kleven et al. 2009) or within other passerines such as the zebra finch (Mossman et al. 2009). For example, Immler et al. (2010) found a positive relationship between flagellum length and sperm velocity before but not after manipulating the social structure in Gouldian finches. Since sperm behavior is likely to be influenced by a range of factors apart from sperm morphology (e.g., seminal fluid; Cornwallis and O'Connor 2009), it seems plausible that an association between sperm form and function is somewhat context-dependent and potentially confounded by (independent) seasonal variation in other ejaculate traits. Overall, it remains to be seen whether direct functional relationships exist between sperm morphology, sperm velocity and sperm number in the red-winged blackbird.

Regardless of whether the multiple ejaculate traits are directly and functionally linked, if their respective seasonal changes are adaptive, they could be hypothesized to indicate differential male investments in ejaculates, potentially in response to nesting synchrony and resulting dynamics in extra-pair copulations and probability of competitive fertilizations (e.g., Westneat and Gray 1998). For example, while large numbers of females are fertilizable simultaneously during the early season, with males suffering little paternity loss due to mate guarding and frequent copulations with the females on their territories (Westneat 1993a, b, 1994),

nesting synchrony decreases as a result of re-nesting or the arrival of secondary females on the breeding site (Robertson 1973; Westneat 1992). In such a scenario, males might invest primarily in sperm quantity early in the season and, when the population becomes asynchronous and the risk of extra-pair copulations increases (Westneat 1993b; Searcy and Yasukawa 1995), shift investments towards sperm quality if sperm quality plays an important role in situations of competitive fertilizations as in other species (e.g., Birkhead et al. 1999; Gage et al. 2004; Malo et al. 2005; Lüpold et al. 2012). Although variation in sperm quality traits in response to differential levels of sperm competition or mating tactics have been documented for a range of taxa (reviewed in Pizzari and Parker 2009), we found no association between the number of fertile females available and any of the ejaculate traits. However, we were unable to account for extra-pair copulations, such that it is difficult to examine to what extent ejaculate characteristics might covary with overall reproductive performance. Additionally, if an association with the risk of extra-pair copulations did exist, we also do not know how the relative investment in sperm quantity and quality might relate to competitive fertilization success.

Conclusions

We reported phenotypic plasticity in multiple ejaculate traits of a temperate, seasonally breeding passerine bird. Whereas seasonal fluctuations in numerous ejaculate traits are well established and often linked to changes in testosterone level, variation in sperm morphology has been overlooked. In comparison with the continuously breeding zebra finch, in which sperm morphology appears to be highly consistent within males, our study indicates that seasonal breeders might be more susceptible to temporal fluctuations in ejaculate characteristics. These data extend our understanding of intra-specific, and particularly intra-male, variation in sperm traits. Further studies are now needed to elucidate the adaptive significance of such variation as well as its underlying mechanisms.

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Ethical standards All fieldwork complies with the current laws of the USA, where the study was performed, and samples of all populations were collected under license of the respective authorities.

Conflict of interest The authors declare that they have no conflict of interest.

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